Molecular phylogeny of three Moroccan *Chalcides*: the *C. manueli*, *C. montanus* and *C. polylepis* clade

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

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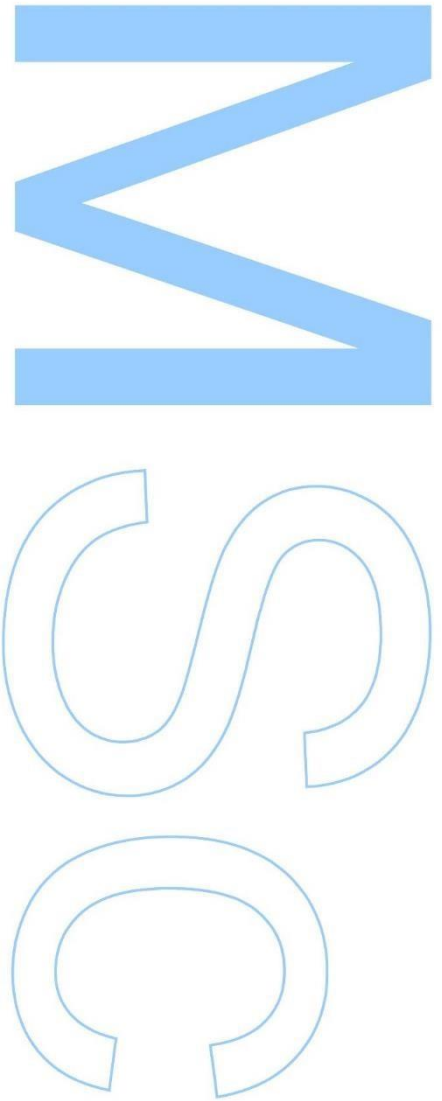

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LIST OF ABBREVIATIONS

Cytb – Cytochrome *b*

ACM4 – Acetylcholinergic Receptor M4

MC1R – Melanocortin 1 Receptor

nuDNA – nuclear DNA

mtDNA – mitochondrial DNA

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ABSTRACT

The Mediterranean Basin, in which Morocco is included, is one of the Global Biodiversity Hotspots. Specific characteristics of Morocco, such as the climate and topography, allow high levels of richness and endemism, particularly in herpetofauna.

The skink genus *Chalcides* comprises 28 species, many of them present in Morocco and surrounding areas, and these are characterized by having elongated bodies and reduced limbs. The taxonomy of *Chalcides* has been revised numerous times, but many species remain difficult to identify in the field, and it seems likely that the current taxonomy still does not fully reflect their evolutionary history.

A previous study regarding the phylogeny for the entire genus based on mtDNA identified four major groups. In this work we investigate the molecular phylogenetic relationships of the three taxa within the "Western clade": *C. manueli, C. polylepis* and *C. montanus*. Mitochondrial and nuclear genes of most of the known populations of these three species were analyzed. Our results indicate that current taxonomy does not reflect genetic diversity.

None of the three currently recognized species were monophyletic. This was not due to mtDNA introgression, as had been previously hypothesised, as nuclear markers were also discordant with current taxonomy. Levels of genetic diversity were quite high, but not as high as typically seen between three different species. Given that the morphological characters used to distinguish the species, primarily size and colour pattern differences, are known to be prone to homoplasy, we propose that the three species are better refered to as a single, albeit quite variable species*, C. polylepis*. This has important conservation implications, as *C. manueli* is currently listed on the IUCN red list as vulnerable.

Further studies are still needed to improve knowledge on the genus. A more efficient sampling effort should be taken into account as well as the detailed assessments on the area of possible sympatry between the three morphological forms of *Chalcides polylepis*. A more thorough study of morphological characters could also turn out to be important in species delimitation in other members of the genus.

KEYWORDS: *Chalcides*, phylogenetic analyses, phylogeography, haplotype networks, mtDNA, MC1R, ACM4

RESUMO

A Bacia do Mediterrâneo, da qual Marrocos faz parte, é um dos Hotspots Globais de Biodiversidade. Características específicas deste país, como o clima e topografia, permitem grandes níveis de riqueza e endemismo, particularmente de herpetofauna.

O género em estudo, *Chalcides* compreende 28 espécies, muitas das quais estão presentes em Marrocos e países adjacentes, sendo caracterizados pelo seu tronco alongado e membros reduzidos. A taxonomia deste género foi revista diversas vezes, e apesar das espécies serem descritas no campo através da sua morfologia, a sua similaridade complica tal feito, parecendo provável que a taxonomia atual não reflita completamente a sua história evolutiva.

Num estudo anterior baseado em DNA mitocondrial, foram identificados a presença de quatro grupos principais. Neste trabalho, o foco de estudo são as relações filogenéticas entre três espécies do "Clado Ocidental": *Chalcides manueli*, *C. polylepis* e *C. montanus*. Genes mitocondriais e nucleares das populações mais conhecidas foram analisados e os resultados indicam que a taxonomia atual não se encontra correta, não refletindo a atual diversidade genética.

Nenhuma das três espécies atualmente reconhecidas é monofilética e tal não é devido a introgressão do mtDNA como foi sugerido anteriormente, visto que os marcadores nucleares também são discordantes com a taxonomia atual. Os níveis de diversidade genética são relativamente elevados mas não tão elevados como seria de esperar entre três espécies diferentes. Tendo em conta que os caracteres morfológicos utilizados na identificação das espécies, nomeadamente tamanho e coloração dorsal, são conhecidos por serem propensos a homeoplasia, propomos que as três espécies sejam apenas consideradas como uma, embora bastante variável, *C. polylepis*. Isto tem implicações relevantes na conservação da espécie, como no caso de *C. manueli*, que até então se encontra listado na IUCN Red List como vulnerável

De futuro os estudos baseados na morfologia de *Chalcides* deverão levar mais em conta a área de simpatria dos três morfotipos de *C. polylepis*. A recolha de indivíduos terá também que ser mais cuidadosa uma vez que estes exemplares de difíceis de encontrar e apanhar. Análises morfológicas mais detalhadas serão também um fator importante na delimitação de espécies.

PALAVRAS CHAVE: *Chalcides*, análise filogenética, filogeografia, redes haplotípicas, DNA mitocondrial, MC1R, ACM4

CHAPTER 1: INTRODUCTION

1. The Scincidae family and the Scincinae subfamily

The Scincidae Family

From the approximate 16 families of lizards, the Scincidae family is the largest and most diverse one, comprising around 149 genera and 1500 species that are widely distributed (Uetz, n.d.). Specimens belonging to this family are often known as skinks and belong to the infraorder of Scincomorpha, a group of families whose members tend to be elongated and have a relatively long snout and flattened skulls. The heads of skinks are usually covered with enlarged plates, termed head shields (seen in Figure 1.1) and osteoderms are frequently present in some or all scales (Glasby, Ross, & Beesley, 1993).

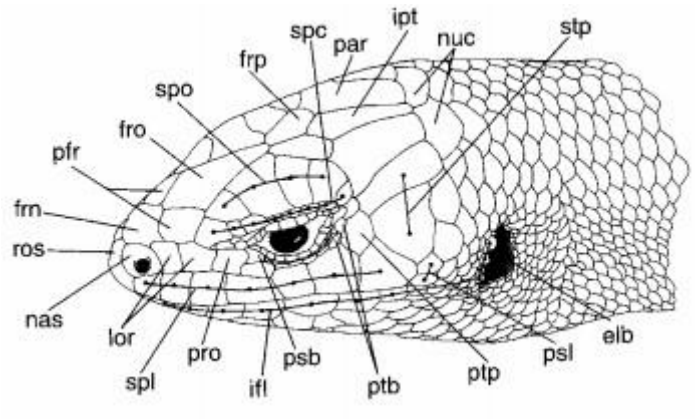

Figure 1.1 – Head of a skink, *Egernia whitii*, showing the enlarged shields and their nomenclature: **elb***, ear lobules;* **frn**, frontonasal; **fro**, frontal; **frp**, frontoparietal; **ifl**, infralabials; **ipt**, interparietal; **lor**, loreals; **nas**, nasal; **nuc**, nuchals; **par**, parietal; **prf**, prefontals; **pro**, preocular; **psb**, presubocular; **psl**, postupralabials; **ptb**, postsubocular; **ptp**, primary temporal; **ros**, rostral; **spc**, supraciliaries; **spl**, supralabials; **spo**, supraoculars; **stp**, secondary temporals. (Glasby et al., 1993).

Typically skinks are diurnal and terrestrial lizards that are active on the surface of the ground or low perches. A significant number of species of the family are secretive or fossorial; this means that they carry out most of their activities underground or protected by leaf litter. However, some are climbers, living in trees and on rocks. This family is present in almost all ecosystems, from tropical forests to desert sand dunes and alpine habitats. There are even some semi-aquatic species that are able to swim and hide underwater when hiding from predators (Daniels 1990). Some of those semiaquatic species inhabit in the intertidal zone, on rocky shores, beaches and mangroves.

Scincids tend to be slightly to significantly elongated lizards with moderate to short limbs and glossy cycloid scales, reinforced by characteristic compounds called osteoderms (see Figure 1.2). Their size varies between 120 to over 350 mm while their weight ranges from 0.4 to over 1000 g. Most skinks have a small head, not very well delineated from the neck, a body square in cross-section with well-developed limbs

bearing graduated toes (4>3>2=5>1) and a long tail (usually more than 120% of the snout-vent length). Depending on whether the species are fossorial or semiaquatic, several differences may appear. In the first case, skinks' head becomes relatively smaller, narrower and wedge-shaped; and their body elongates while their limbs become smaller, in some species the number of digits decreases as well (Glasby et al., 1993).

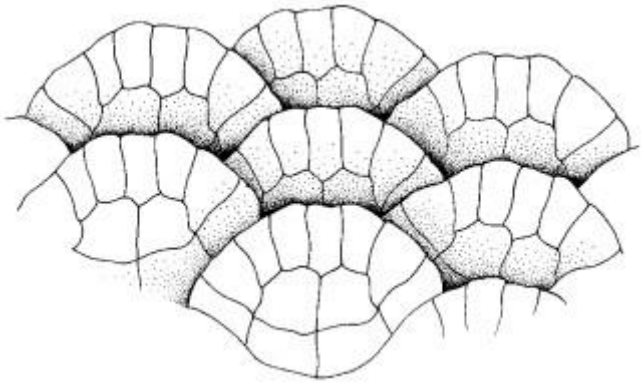

Figure 1.2 – Osteoderms from the dorsal scales of *Eremiascincus fasciolatus* showing the compound structure characteristic of skinks (Glasby et al., 1993).

Sexual dimorphism is usual in skinks, with females having longer bodies than males, and proportionally narrower heads and shorter appendages, even though these differences may be very subtle sometimes (Glasby et al., 1993).

When focusing on colour combination and colour patterns, it seems that terrestrial species have a tendency to have longitudinal patterns, especially grass-dwellers; while transverse barring is more common in secretive shade and litter-dwelling species. A combination of grey-brown dorsum with blackish sides, form the colour pattern for surface-dwelling woodland species whereas climbing species, and rock dwellers, are usually spotted or mottled, with almost no trace of linear pattern elements. Borrower species tend to have little to none pattern however they may present bright colours in the tail, belly or head. Sexual dichromatism may also be present although it is not very common (Glasby et al., 1993).

Skinks typically have relatively large scales, with a maximum of 40 longitudinal rows of body scales. The number of longitudinal rows of scales is somewhat related to whether they are dwelling species, usually with less than 28 rows of scales along the body, or climbing species, usually with 34 or more rows of scales along the body, for example. This tends to enhance "armour-plating" in the burrowing/litter dwelling species and softness and flexibility in the climber species (Glasby et al., 1993).

Usually, skinks' scales have a smooth surface, dorsally matted and laterally and ventrally polished. In some taxa, keels or striations may be present. Renous and Gase (1989) studied the micro-ornamentations in lizard scales, including in skink species, and demonstrated that the ornamentation varies according to the degree of contact between

a region of the body surface and the substrate. However, in skinks, this has not yet been used as a phylogenetically informative character (Glasby et al., 1993). Regarding skinshedding, skinks seem to periodically shed a complete outer epidermal layer, although it does not shed as a unique patch. Moreover, diurnal skinks have a black lining to the cranial and body cavities which it is absent in nocturnal skinks and fossorial species. Porter (1967) explained the black colouring as providing an internal shield blocking high energy ultra-violet radiation which could cause tissue damage to the skink.

It seems that skinks' speed is related to limb length in normally proportioned species. However, in reduced limb species, the tail and sinuosity of the body become significant in their locomotion. Normally limbed species can run faster with a chopped tail but the same does not occur in reduced-limbed species, decreasing as well their swimming speed (Glasby et al., 1993).

The Scincinae Subfamily

The Scincidae family has seven recognised subfamilies: the Acontinae, Egerniinae, Eugonglynae, Lygosominae, Mabuyinae, Sphenomorphinae and the paraphyletic Scincinae (Uetz, n.d.). It comprises more than 1300 species (Schmitz et al. 2005; Whiting,2003), which is more than 25% of the world's lizard diversity (Whiting, 2003). The Scincinae subfamily has around thirty three genera, *Amphiglossus*, *Androngo*, *Ateuchosaurus*, *Barkudia*, *Brachymeles*, *Chabanaudia*, *Chalcides*, *Eumeces*, *Feylinia*, *Gongylomorphus*, *Hakaria*, *Janetaescincus*, *Jarujinia*, *Madascincus*, *Melanoseps*, *Mesoscincus*, *Nessia*, *Ophiomorus*, *Pamelaescincus*, *Paracontias*, *Plestiodon*, *Proscelotes*, *Pseudoacontias*, *Pygomeles*, *Scelotes*, *Scincopus*, *Scincus*, *Scolecoseps*, *Sepsina*, *Sepsophis*, *Sirenoscincus*, *Typhlacontias* and *Voeltzkowia* (Whiting, 2003).

This subfamily is distributed worldwide (Schmitz et al., 2005; Whiting, 2003), displaying a remarkable array of morphological variation and characterized by the repetition of the evolution of body elongation and limb reduction (Schmitz et al., 2005). Several fossorial/semi-fossorial species in this family have completely or severely reduced their limb size (Schmitz et al., 2005), and limb reduction has evolved multiple times across multiple genera, making it difficult to estimate evolutionary relationships based on morphology (Crottini et al., 2009; Schmitz et al., 2005).

The Genus *Chalcides*

Taxonomy and Systematics

The genus *Chalcides* was first described by Laurenti in 1768. It currently comprises 28 species (Carranza, Arnold, Geniez, Roca & Mateo, 2008) and is considered to be a fairly primitive clade within the subfamily Scincinae (Vincenzo Caputo & Mellado, 1992; Vincenzo Caputo, 2004).

George A. Boulenger was the first herpetologist working with this genus and he recognized six varieties with the widespread *C. ocellatus* describing as well *C. bottegi* from Ethiopia. Meanwhile, Boulenger's youngest son, Edward, expanded his father's research by arguing against the recognition of *C. bottegi* as a different species and summarizing the morphology and colour patterns of eight different forms of *C. ocellatus*. Thanks to this work, other North African and insular species of the genus received more attention (Vincenzo Caputo, Lanza & Palmieri, 1995; Vincenzo Caputo & Mellado, 1992; Carranza et al., 2008; Schleich, Kästle, & Kabisch, 1996) but still little was published on sub-Saharan *Chalcides* (Caputo et al., 1995).

The genus *Chalcides* traditionally has a problematic taxonomic history exacerbated by the fact that specimens of many species of this genus are generally rare and hard to collect (Greenbaum, Campbell, & Raxworthy, 2006). Due to convergence of several morphological traits (Schleich et al., 1996), no robust morphological phylogenies are available and there still large uncertainties also at the molecular level (Carranza et al., 2008). Even though several different authors (such as: G. Boulenger 1887, 1890, 1896, 1898; E. Boulenger 1920; Parker 1932; Angel 1936; Loveridge 1936; Angel and Lothe 1938; Lanza 1954; Lanza 1957; Laurent and Gans 1965; Lanza and Carfi 1968; Drewes 1972; Pasteur 1981; Welch 1982; La Berre 1989; Caputo 1993; Caputo *et al.* 1995; Mateo *et al.* 1995; Spawls *et al.* 2002; Greenbaum 2005; Greenbaum *et al.* 2006) studied the taxonomy of the species, the same species were catalogued under different specific names. This has led to the existence of a large number of miscataloged specimens hosted in Museum collection, exacerbating the confusion on their taxonomy and systematics. Recently, this genus has been the subject of a detailed morphological and molecular analyses (Carranza et al., 2008; Hoser, Road, & Orchards, 2012) identifying four distinct clades (Carranza et al., 2008).

Biology and Morphology

Chalcides are small to medium-sized elongated lizards often with shortened to reduced limbs (Vincenzo Caputo & Mellado, 1992; Carranza et al., 2008; Schleich et al., 1996) and smooth shiny scales (Hoser et al., 2012), adapted to subterranean lifestyles (Carranza et al., 2008). During the process of evolution, the limb-size and number of digits

in several species was apparently reduced multiple times and the body shape became more elongated (Vincenzo Caputo, 2004; Hoser et al., 2012; Schleich et al., 1996). They present a lower eyelid with an undivided transparent disk and the nostril pierced between an emargination of the rostral and a very small nasal. The supranasals are present, but prefrontals and frontoparientals are absent (Schleich et al., 1996).

Their head is generally small to medium and the neck relatively thick; they feed on a variety of items, mainly including small invertebrates such as worms, small snails, insect larvae, ants, termites, grasshoppers, crickets and coleoptera (Hoser et al., 2012; Schleich et al., 1996).

These species have a mixed type spermatogenesis with a short vernal period. They are ovoviviparous or viviparous having clutches of 1 to 2 eggs (Schleich et al., 1996).

Available Phylogenies

Carranza *et al.* (2008) states that *Chalcides* have a probable origin in Morocco, and from there, two lineages of *Chalcides* invaded the Canary Islands while at least five main lineages invaded and colonized southern Europe. Another five should have spread across northern Africa as far as southwest Asia.

Chalcides would have diversified into four main clades around 10 Ma: the Grassswimming clade, the Northern clade, the *C. ocellatus* clade and the Western Clade. *C. manueli*, *C. montanus* and *C. polylepis* are part of the Western clade as well as *C. sphenopsiformis*, *C. viridanus*, *C. coeruleopuntatus*, *C. s. sexlineatus*, *C. s. bistriatus*, *C. simony*, *C. m. mionecton* and *C. m. trifaciatus* (Carranza et al., 2008). The phylogeny he suggested for the *Chalcides* group is available in Figure 1.3.

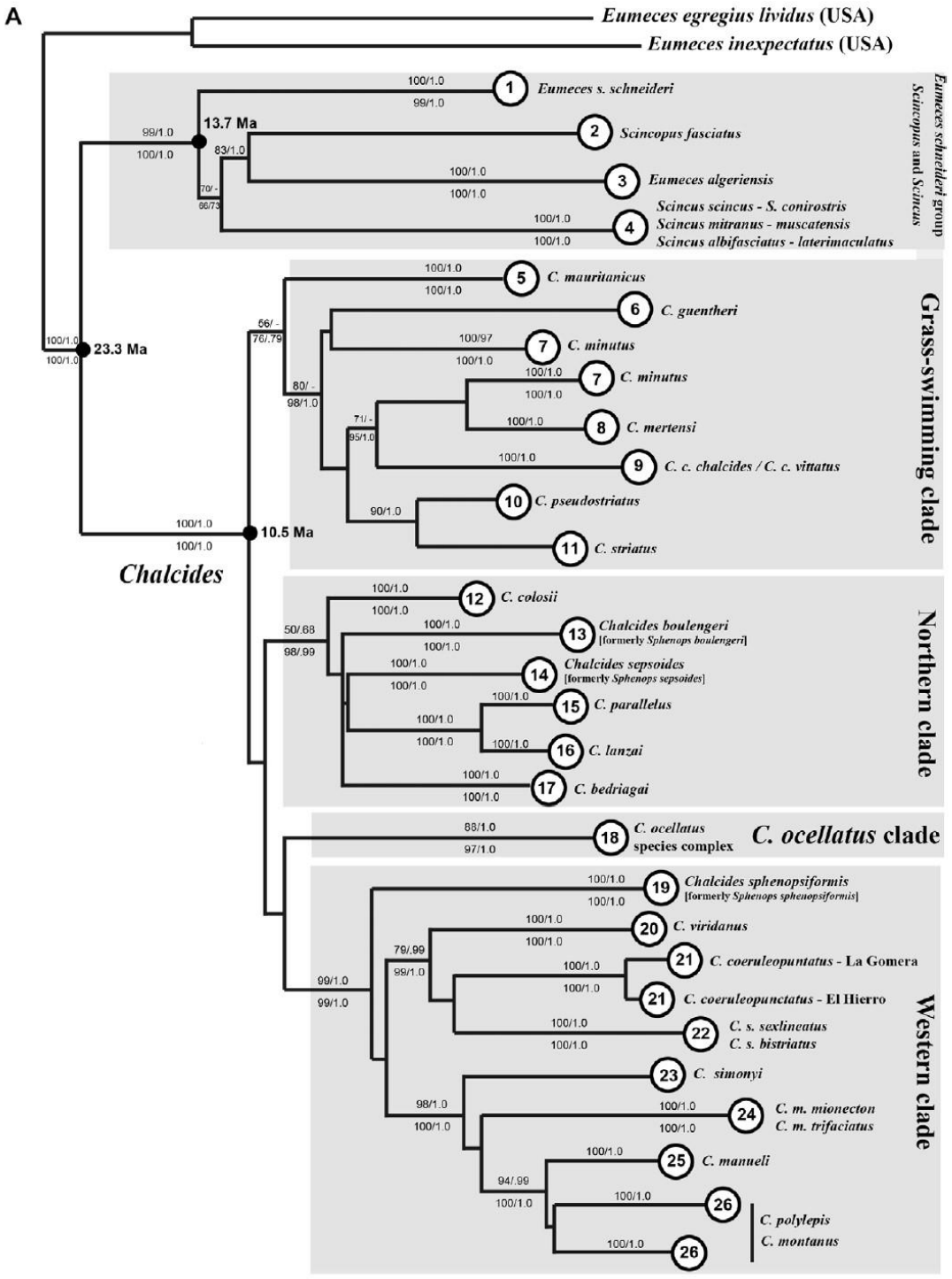

Figure 1.3 – North African *Chalcides*' phylogeny by S. Carranza (Carranza *et al.* 2008).

Distribution and biogeography

This genus has a wide distribution, being present in Macaronesia, Northern Africa and Southern Europe and extending to the East, including countries such as Somalia, Kenya, Turkey, Iraq, Arabia, Iran and Pakistan (Vincenzo Caputo & Mellado, 1992; Vincenzo Caputo, 2004; Carranza et al., 2008; Giovannotti, Cerioni, Kalboussi, Aprea, & Caputo, 2007; Hoser et al., 2012; Schleich et al., 1996).

Calibrated molecular phylogenies suggest that *Chalcides striatus* colonized southwest Europe around 2.6 Ma, while, *C. chalcides* colonized mainland Italy around 1.4 Ma, both across water. More recently, *C. c. vittatus* reached Sardinia and *C. guentheri* spread 1200 km further east to Israel. Around 5 Ma, *C. boulengeri* and *C. sepsoides* spread east through sandy habitats (north of the Sahara) until they reached Egypt, while *C. bedriagai* invaded and diversified in Spain, probably during the Messinian period when the Mediterranean was dry. *C. ocellatus* lineages have independently invaded Malta and Sardinia from Tunisia and the southwest Arabia; *C. o. humilis* as well as *C. delislei*, from the Western clade, appears to have spread over 4000 km through the Sahel, south of the Sahara, probably in the Pleistocene. Canary Islands were colonized twice: the first around 5 Ma by *C. simony*, and the other about 7 Ma by the ancestor of *C. viridanus* + *C. sexlineatus* (Carranza et al., 2008).

2. The Study site

Morocco

Geography

Morocco is a country in the Maghreb region of North Africa, characterized by a rugged mountainous interior and wide desert areas. The Atlas Mountains are located mainly in the centre and the south of the country while the Rif Mountains are located in the north of the country. Together with Spain and France is the only country with both Atlantic and Mediterranean coastlines (Bons & Geniez, 1996).

Climate

The coastal plains are rich and constitute the backbone of the agricultural environment, especially in the northern areas. While the Atlas Mountains are important in the climate definition, dividing the country into two different bioclimatic regions (Bons & Geniez, 1996):

- The north and west Morocco have a Mediterranean climate, with hot dry summers and mild winters with irregular and frequent precipitations. This area is part of the semi-arid and sub-humid bioclimatic zones (Bons & Geniez, 1996);
- The southern part of Morocco as well as the east, contrary to the rest, have a Saharan climate, with an arid bioclimate. In these regions, summer are dry and with exceptionally high temperatures while the winters are cool or cold, except for the Atlantic coast, where precipitation rarely occurs, only in the winter (Bons & Geniez, 1996).

In general, Morocco is a very windy country, having more than 20 windy days per month, with the winter months being windier than the summer ones. Regarding precipitation, in most parts of the country receive a volume of less than 400 mm of rain per year. However, this volume changes from one area to another. For instance, in the Atlas Mountains, Rif, Tingis peninsula and the Gharb receive over 800 mm annual rainfall, 1400 mm in the summits of the Rif; whereas in the Sahara under 200 mm is registered. In these higher areas of the country, a mean of 10 days (maximum of 20) of snow per year are recorded (Bons & Geniez, 1996).

The temperature range in Morocco varies between <0ºC and 60ºC depending on the area, with the lowest temperatures present in the highest parts of the Atlas Mountains and Eastern High Plateaus, whereas the highest temperatures can be reached in the Saharan climate. The coastal area has mild to very mild winters and tempered summers due to the proximity of the sea (Bons & Geniez, 1996).

Such variation has led to a great variety of habitats, which in turn hosts high biodiversity levels.

Herpetofauna of Morocco

Biodiversity is not homogenously distributed around the world and currently around 25 biodiversity hotspots have been identified, with the Mediterranean Basin, in including Morocco, being one of them (Keppel et al., 2012; Médail & Quézel, 1999; Myers, Mittermeier, Mittermeier, da Fonseca, & Kent, 2000).

Morocco is one of the richest country in North Africa and the Western Mediterranean area regarding herpetofauna (along with Algeria), having more than 104 species from which 23 are endemic to the area (Bons and Geniez 1996). This is the result of a combination of different factors. Morocco can to some extent be considered "isolated" due to its natural barriers: from the north by the Mediterranean Sea, from the west by the Atlantic Ocean, from the south by the Sahara and from the east by the Oued Moulouya depression which is particularly arid, constituting a barrier against animal population exchanges. Even within the country there are many other factors contributing to the presence of such a high number of endemisms: geographic entities such as the Atlas Mountains, the Rif Mountains and the Atlantic plains for example, are insuperable barriers for a large number of species that cannot find favourable conditions. Also, these mountains separate the country into two different bioclimatic zones: the Mediterranean Morocco and the Saharan Morocco. It is also the climatic differences between different parts of the country that lead to the presence of the numerous endemisms (Bons & Geniez, 1996).

The three species which are the focus of this thesis: *Chalcides manueli*, *C. montanus* and *C. polylepis*, are all Moroccan endemic (Bons & Geniez, 1996).

Chalcides manueli, C. montanus and C. polylepis

Chalcides manueli

Taxonomy and Systematics

Chalcides manueli (Figure 1.4) was first described by Hediger in 1935, in dedication of Albert Manuel of Rabat who helped him organizing the expedition and helped in the fieldwork. It was originally considered as a subspecies of *C. ocellatus* (Bons & Geniez, 1996), but it is currently been considered a different species based on considerable differences in morphology and colouration (Bons & Geniez, 1996; Vincenzo Caputo & Mellado, 1992; Schleich et al., 1996). Furthermore, it is morphological very similar and is found in close geographic proximity to *C. ocellatus* and *C. montanus* without having any known intermediate forms (Bons & Geniez, 1996).

Figure 1.4 – *Chalcides manueli* (from: https://www.flickr.com/photos/alberto_herpetology/14016912974/in/photolistnmCkTm-njLg6U-nmiBMv-njxtqu).

Morphology

C. manueli presents a uniformly brown to dark olive brown colouration, lacking a conspicuous dorsal pattern, and no ocelli, being very similar in this respect to *C. colosii* (Bons & Geniez, 1996; Schleich et al., 1996). It has some black spots in the neck, which do not pass beyond the foreleg insertion (Schleich et al., 1996).

C. manueli has a snout-vent length (SVL) of around 65.5 mm to 75.9 m and 28 to 31 scales around midtruck (Schleich et al., 1996).

Habitat and Ecology

Chalcides manueli is present in the foots of the western slopes of the High Atlas, with arid and semiarid zones with temperate or warm winters (Bons & Geniez, 1996).

Distribution

This species is endemic from Morocco, and is present in the base of the western slopes of the High Atlas, from Dar Mzoudi to Taroudannt, including coastal areas such as Essaouira and Agadir, and in arid and semi-arid areas with temperate or warm winters (Bons & Geniez, 1996; Schleich et al., 1996). More recently, Carranza *et al.* (2008) included samples from Sidi Ifni (slightly to the south) to the previously known range. The species distribution and location of the used samples is represented in the map on Figure 1.5.

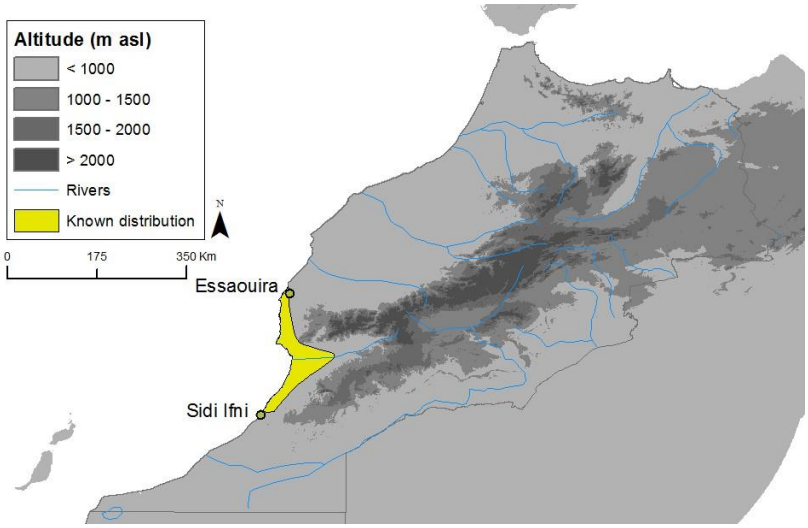

Figure 1.5 – Known location of *Chalcides manueli* (yellow area) and samples used for the study (yellow dots).

Chalcides montanus

Taxonomy and Systematics

Chalcides montanus was first described by Werner in 1931 as a subspecies of *C. ocellatus* but was later raised to the species level (Vincenzo Caputo & Mellado, 1992). Mateo *et al.* (1995) had considered two subspecies of *C. montanus*: *Chalcides montanus montanus* and *Chalcides montanus lanzai* (previously described by Pasteur in 1967 as *Chalcides ocellatus lanzai*) but later, the latter was again considered an independent species (Bons & Geniez, 1996; Carranza et al., 2008).

Morphology

C. montanus is overall beige to brown with a yellow venter, presenting four longitudinal white lines which begin at the nuchals and continue over at least 15 scales (Figure 1.6 A). This striation often fades in the posterior scales, and there are no ocelli present behind the hindlegs. In each side there is a distinctly marked light dorsolateral stripe without ocelli (Schleich et al., 1996). Juveniles and subadults of this species present an orange colouration of the snout and tail (Figure 1.6 B), making their identification easier. This colouration may also prevail in adulthood (Bons & Geniez, 1996).

C. montanus has a SVL of around 72.5 mm to 99 mm and 28 to 32 scales around midtrunk (Schleich et al., 1996).

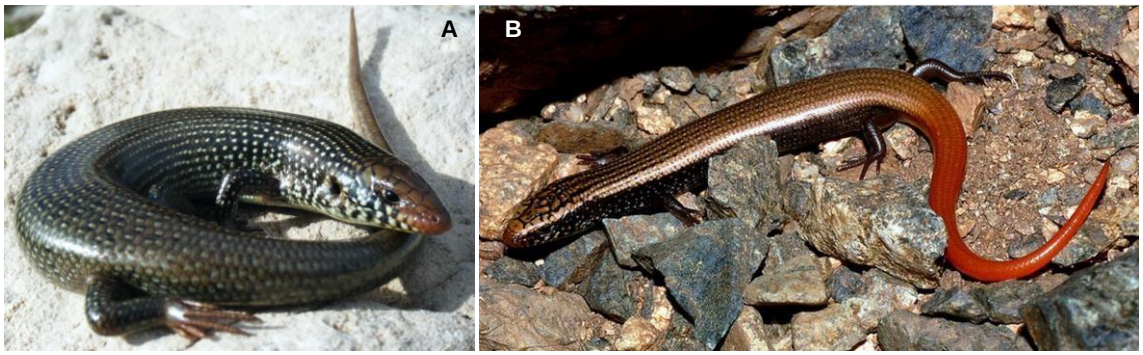

Figure 1.6 – *Chalcides montanus* adult (A) (from: https://www.flickr.com/photos/82470440@N06/8404230760/in/photolist-akXadx-6X5wsx-dNDT1m) and juvenile (B) (from: https://www.flickr.com/photos/gabrimtnezmarmol/3905074027/sizes/z/).

Habitat and Ecology

This species is present in cold and moist mountain regions with low bushes around 1500 m to 2830 m high (Bons & Geniez, 1996; Schleich et al., 1996). As such, this species hibernates for almost half the year due to the weather conditions (humid and upper sub-humid bioclimatic areas with cold winters) (Bons & Geniez, 1996; Schleich et al., 1996).

Distribution

C. montanus is endemic to Morocco, and is present in the High and Middle parts of the High Atlas (such as Tizi Tachedirt, 2200 m, and Tizi Tamatert, 2400 m). With the recognition of *C. lanzai* as a full species, very few localities are known, and the species appear to be rare or sporadic in the High Atlas. It is present in localities including the Toubkal Masssive, Goulmima and Missour, with these last two presenting a slightly different colour, reaching its southern limits on Djbel Siroua (Bons & Geniez, 1996; Schleich et al., 1996). The species distribution and location of the used samples is represented in the map of Figure 1.7.

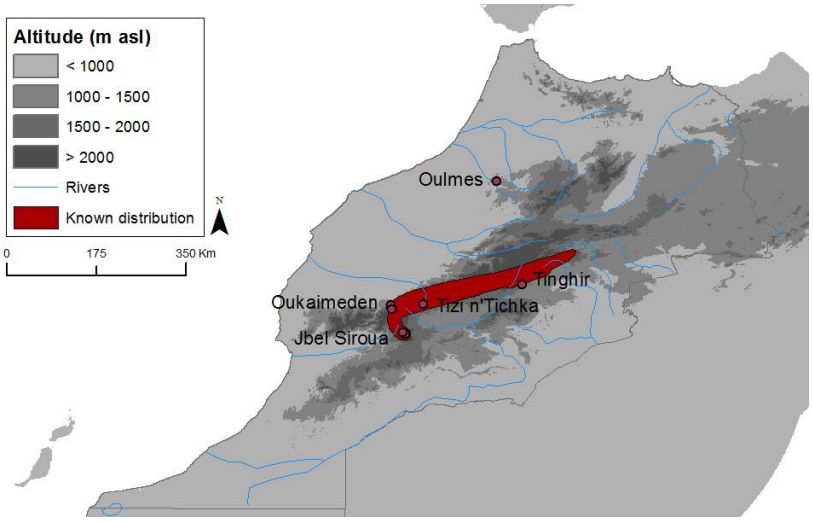

Figure 1.7 – Known location of *Chalcides montanus* (red area) and samples used for the study (red dots).

Chalcides polylepis

Taxonomy and Systematics

Chalcides polylepis was first described by Boulenger in 1890 (Bons & Geniez, 1996) as *Chalcides ocellatus* var. *polylepis* but in 1957, Lanza recognized it as a full species, *Chalcides polylepis.*

Morphology

The general colour of *Chalcides polylepis* (Figure1.8) is yellowish brown or grey, dark grey to black, with ocelli forming parallel longitudinal and transversal white lines on the dorsal area and flanks. The central spots of the ocelli are usually round and the pattern change with increasing age. Juveniles of *C. polylepis* are usually uniformly ocellated, while adults have only traces of ocelli left in middorsal rows (Schleich et al., 1996).

C. polylepis has a robust head and body (SVL around 150 mm), being much larger than *C. montanus* (SVL of around 72.5-99 mm) and *C. manueli* (SVL of around 65.5-75.9 m) but smaller than *C. ocellatus* (SVL of around 130-140 mm). It presents around 34 to 40 scales around midtrunk (Schleich et al., 1996).

Figure 1.8 – *Chalcides polylepis* from Sidi Ifni, May 2013 (picture by Daniele Salvi, Morocco 2013).

Habitat and Ecology

C. polylepis is present in hillsides or flat areas, under stones and nearby bushes, walls and in refuse dumps, from coastal areas to mountains of up to 2000 m high. It uses as refuges both buried stones and plant thickets (Schleich et al., 1996).

This species tends to stay close to its refuge. From mid-June onwards, *C. polylepis* become more secretive and estivate until autumn, hibernating from November until the beginning of March under stones or roots (Schleich et al., 1996).

Distribution

This species is endemic to Morocco, (Bons & Geniez, 1996), being most widespread than *C. manueli* and *C. montanus*. It can be find inside the triangle Tangier (Boettger in 1883) – Taza (Werner in 1931) – Laâyoune (Valverde in 1957), and reaches 1950 m high on Djbel Ighnayene in the central High Atlas. *Chalcides polylepis* is present in a great variety of habitats being complete absent only on dense woodlands (Bons & Geniez, 1996). During fieldwork in 2013, in which I took part, a new locality at Sidi Ifni (Figure 1.9) was reported (Rosado & Al, n.d.). This is important, as *C. manueli* was also reported from Sidi Ifni recently (Carranza et al., 2008). The species distribution and location of the used samples is represented in the map of Figure 2.6.

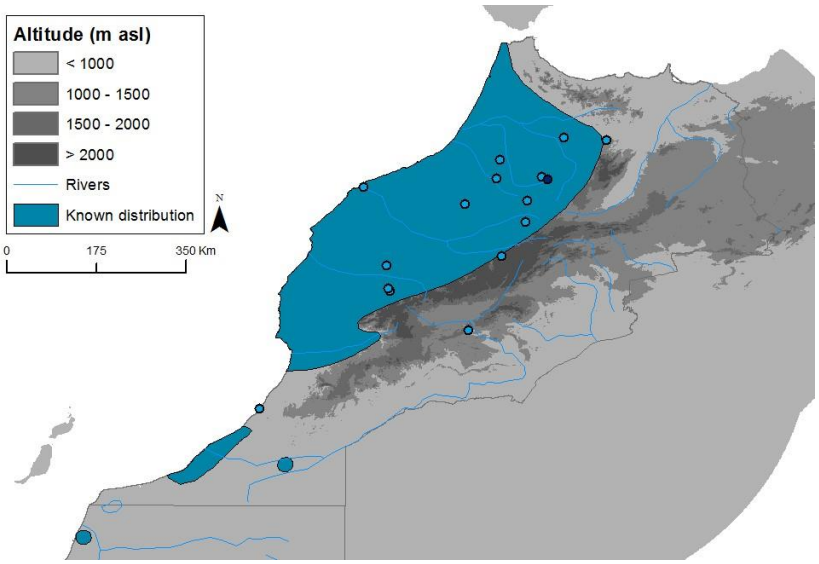

Figure 1.9 – Known location of *Chalcides polylepis* (blue area) and samples used for the study (light blue dots). A sample of *Chalcides sp. aff. Polylepis* is also represented by a dark blue dot.

3. Molecular phylogeny and phylogeography

Molecular phylogeny history and applications

Phylogenetics, from the Greek /faɪlɵdʒɪˈnɛtɪks/ is the study of evolutionary relationships among groups of organisms, which are typically estimated through analysis of molecular sequencing data and/or morphological data matrices. Phylogenetic analyses result in a hypothesis about the evolutionary history of taxonomic groups: their phylogeny.

It was in the mid-1960s that molecular evolutionists started arguing that molecular evidence was a more reliable tool than morphology, in which classical systematics had previously relied (Suárez-Díaz & Anaya-Muñoz, 2008). The theoretical frameworks for molecular systematics began to appear in the works of Emile Zuckerkandl, Emmanuel Margoliash, Linus Pauling, and Walter M. Fitch, which contrasted with the works of Ernst Mayr, Theodosius Dobzhansky and George G. Simpson, who saw molecular information as a single character to be considered in a battery of usable characters (Suárez-Díaz & Anaya-Muñoz, 2008).

DNA and proteins started then to be considered the optimal markers for estimating the evolutionary history of the species, since genes were considered to give better explanations on the evolutionary biology of species as well as they were better suited for quantitative and statistical analysis than any other type of evidence used so far (Suárez-Díaz & Anaya-Muñoz, 2008). In general, closely related organisms have high degree of agreement in their molecular structure, while DNA, RNA and proteins from distantly related organisms show a pattern of dissimilarity. A further advantage of molecular data is that it may be possible to apply a “molecular clock” to date times of taxa divergences.

Molecular phylogenetics alongside with the use of molecular data, taxonomy and biogeography, constitute the backbone of molecular systematics. The most common applications of molecular phylogeny is the study of phylogenetic relationships of different taxa.

Phylogeography history and applications

Before phylogeographies was proposed as new field of investigation, scientists focused on the study of intraspecific (microevolutionary) genetic processes, such as population genetics, that dealt with changes in population allele frequencies resulting from mutation or genetic drift, etc. Separately, others dedicated their time in analysing supraspecific (macroevolutionary) genetic patterns such as phylogenetics and systematics, dealing mostly with evolutionary relationships of species and higher taxa. Population geneticists used to have strong background on statistics, mathematics, ecology or population demography while systematics on museums or field work on a particular taxonomic group. Following Avise et al (2009) in order to have a temporal

evolutionary continuum, both population genetics and phylogenetic biology should be studied together. Thus the strength of "phylogeography" was that it attempted to bridge these two previously distinct fields of research (Avise, 2009).

Phylogeography is the study of the historical processes that may be responsible for the current geographic distribution of organisms, describing geographically structured genetic signals within and among species. When focusing in a species' past, biogeography alongside with its gene genealogy, population genetics and phylogenetics can be set apart from phylogeography. Past events such as population expansion, bottlenecks, vicariance and migration can be inferred

Phylogeography is a relatively new discipline that deals with the spatial arrangements of genetic lineages, especially within and among closely related species; having a special focus on population history and demography. Since it was outlined in 1987, phylogeography has grown exponentially (Avise, 2009). It could be considered a branch of population genetics with a special focus on genealogy (not just allele frequencies), as well as a branch of phylogenetic biology, since it deals with genealogy (see Figure 1.10) (Avise, 2009).

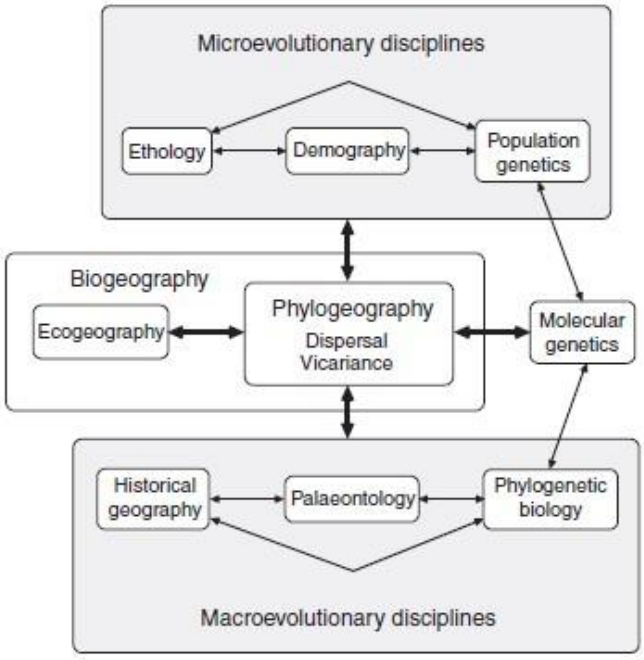

Figure 1.10 – The general place of phylogeography, and some of its empirical and conceptual bridging functions, within the biodiversity sciences (Avise, 2009).

Aims of the study

Species delimitation has been an important and controversial area within evolutionary biology. Species boundaries have been defined using morphological characteristics but more recently the use of genetic approaches helped to define this delimitation.

The *Chalcides* complex has been investigated over a long period and, still, the relationships among *C. manueli*, *C. montanus* and *C. polylepis* remains unclear.

The aim of this thesis is to contribute to the better understanding of the phylogeny of this complex of species, using only genetic analysis since the number of specimens available for morphological analysis was insufficient. To do this I analysed the sequences of homologous DNA fragments of four different genes: 12S rRNA, Cytb (Cytochrome *b*), MC1R (Melanocortin 1 Receptor), and ACM4 (Acetylcholinergic Receptor M4) and construct phylogenetic trees and haplotype networks in order to have the maximum information possible. Genetic distances are also calculated in order to understand the variation between and within each of the obtained groups.

CHAPTER 2: MATERIAL AND METHODS

1. Sample Collection

Tissue samples were collected during field expeditions to Morocco from 2000 to 2014. Specimens were actively searched for under rocks and bushes and were caught by hand, and when a specimen was found and caught, its tail tip was collected and stored in 96% ethanol after which, the individual was released in the same place of collection. GPS coordinates of the sampling site were recorded and photos of the individual taken. Of those caught in the fieldtrip of May 2013, sex, age and size were also recorded (2 samples, one *Chalcides montanus* and one *Chalcides polylepis*). Due to this limited number of samples, morphological variation could not be assessed.

The list of the 61 analysed samples was completed with additional material provided by collaborators. In total 17 samples assigned to *C. polylepis* coming from 10 km NW Marrakesh, 18 km Marrakesh, Anergui, Azemmour, Azrou, Dar Caïd Ouriki, Hamriyah, Khémisset, Marrakesh, Medium Atlas, Oulmes, Sidi Azigza, Sidi Ifni, Sidi Yahya and Taza, 1 sample assigned to *C. sp. aff. polylepis* from Mischliffen, 1 sample assigned to *C. sp.* From Sidi Chiker, 29 samples assign to *C. montanus* from Jbel Siroua, Oukaimeden, Oulmes, Tinghir and Tizin Tichka and 7 samples assign to *C. manueli* collected at Essaouira and Sidi Ifni were analysed (See Table 2.1 for additional details). Four individuals of *C. lanzai* three from Azrou and one from Mischliffen and two individuals of *C. mionecton* from Guelmine were originally intended to be used as outgroups for the molecular phylogenetic analyses. However, for the final analysis in MrBayes, the *C. lanzai* individuals were removed as they were too divergent from the other species and resulted in long branches that might cause taxonomic artefacts. The distribution of the 61 samples used is in Figure 2.1.

Table 2.1 - Information of the samples used in the analysis (Specimen code, genus, species, country of collection, locality of collection, GPS coordinates, elevation and presence (x) or absence (-) of sequence per sample, per gene).

Extraction Code	Specimen Code	Genus	Species	Country	Locality	Latitude	Longitude	Elevation	12S	MC1R	Cytb	ACM4
IT0001	DB944	*Chalcides*	*lanzai*	Morocco	Azrou	33.41905167	-5.17841	1811.2 m	x	x	x	x
IT0002	DB945	*Chalcides*	*lanzai*	Morocco	Azrou	33.41902167	-5.178451667	1793.2 m	x	x	x	x
IT0003	DB1105	*Chalcides*	*montanus*	Morocco	Jbel Siroua	30.74262833	-7.610103333	2359.9 m	x	x	x	x
IT0004	DB1106	*Chalcides*	*montanus*	Morocco	Jbel Siroua	30.74258333	-7.609996667	2350.7 m	x	x	x	x
IT0005	DB1108	*Chalcides*	*montanus*	Morocco	Jbel Siroua	30.74262833	-7.610103333	2359.9 m	x	x	x	x
IT0006	DB87	*Chalcides*	*polylepis*	Morocco	Sidi Yahya	32.66195	-5.499433333	1832 m	x	x	x	x
IT0007	DB6858	*Chalcides*	*polylepis*	Morocco	Anergui	32.071035	-5.922862	1607 m	x	x	x	x
IT0008	DB15497	*Chalcides*	*lanzai*	Morocco	Mischliffen	33.40556	-5.10297	2055 m	x	x	x	x
IT0009	DB15533	*Chalcides*	*polylepis*	Morocco	Hamriyah	34.1485	-4.82867	383 m	x	x	x	x
IT0011	DB1118	*Chalcides*	*montanus*	Morocco	Jbel Siroua	30.74262833	-7.610103333	2359.9 m	x	x	x	x
IT0012	DB1120	*Chalcides*	*montanus*	Morocco	Jbel Siroua	30.74262833	-7.610103333	2359.9 m	x	x	x	x
IT0013	DB1639	*Chalcides*	*montanus*	Morocco	Jbel Siroua	30.77671	-7.652988333	2560 m	x	x	x	x
IT0014	DB1686	*Chalcides*	*lanzai*	Morocco	Azrou	33.493033	-5.148317	1679 m	x	_	x	x
IT0015	DB3335	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.209249	-7.852882	2596 m	x	x	x	x
IT0016	DB20160	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.20794	-7.85097	2589 m	x	x	x	x
IT0017	DB20021	*Chalcides*	*polylepis*	Morocco	Sidi Ifni	29.38717	-10.17204	2 m	x	x	_	x
IT0022	DB454	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.20641667	-7.85975	2640 m	x	x	x	x
IT0024	DB1417	*Chalcides*	*sp.*	Morocco	Sidi-Chiker	31.74957833	-8.738441667	200 m	x	x	x	x
IT0025	DB11028	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.20843	-7.86078	2722 m	x	x	x	x
IT0026	DB3931	*Chalcides*	*sp. aff. polylepis*	Morocco	Mischliffen	33.40543333	-5.103316667	2057 m	x	_	_	_
IT0027	DB3949	*Chalcides*	*polylepis*	Morocco	Azrou	33.461268	-5.206361	1287 m	x	x	x	x
IT0028	DB4199	*Chalcides*	*mionecton*	Morocco	Guelmine	28.99783333	-10.052733	305 m	x	x	x	x

IT0029	DB4200	*Chalcides*	*mionecton*	Morocco	Guelmine	28.99783333	-10.052733	305 m	x	x	x	x
IT0030	DB4237	*Chalcides*	*polylepis*	Morocco	Taza	34.10428333	-4.072483333	1460 m	x	x	x	x
IT0031	DB9200	*Chalcides*	*polylepis*	Morocco	Khémisset	33.760237	-5.953675	178 m	x	x	x	x
IT0032	C4491 (1)	*Chalcides*	*polylepis*	Morocco	Medium Atlas	32.973778	-6.568083	891 m	x	x	x	x
IT0033	DB1117	*Chalcides*	*montanus*	Morocco	Jbel Siroua	30.74262833	-7.610103333	2359.9 m	x	x	_	_
IT0034	DB1478	*Chalcides*	*montanus*	Morocco	Jbel Siroua	30.77671	-7.652988333	2561 m	x	x	x	x
IT0035	DB11325	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.19943	-7.85071	2804 m	x	x	_	x
IT0036	DB1691	*Chalcides*	*polylepis*	Morocco	Azrou	33.032133	-5.464417	1491 m	x	x	_	_
IT0037	E140373	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.255869	-7.867102	1791 m	x	x	_	x
IT0038	E140374	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.255869	-7.867102	1791 m	x	x	_	_
IT0039	E14124.1	*Chalcides*	*polylepis*	Morocco	Marrakesh	31.899	-7.939	520 m	x	x	_	x
IT0041	E25061.1	*Chalcides*	*manueli*	Morocco	Sidi Ifni	29.3865	-10.16799	15 m	x	x	x	x
IT0042	E25061.21	*Chalcides*	*polylepis*	Morocco	Sidi Azigza	30.7658	-6.4956	1294 m	x	x	x	x
IT0043	E25061.28	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.255869	-7.867102	1791 m	x	x	_	x
IT0044	E25061.29	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.255869	-7.867102	1791 m	x	_	_	_
IT0045	E28061.	*Chalcides*	*polylepis*	Morocco	Azemmour	33.285068	-8.34795	29 m	x	_	_	_
IT0046	E28061.3	*Chalcides*	*polylepis*	Morocco	Oulmes	33.42173	-6.005382	1175 m	x	_	_	_
IT0047	E4113.23	*Chalcides*	*manueli*	Morocco	Sidi Ifni	29.3865	-10.16799	15 m	x	x	x	_
IT0049	E60281	*Chalcides*	*manueli*	Morocco	Essaouira	31.515660	-9.65402	131 m	x	x	x	x
IT0050	E602811	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.201369	-7.865269	2693 m	x	x	_	_

IT0051	E6028.18	*Chalcides*	*polylepis*	Morocco	18 Km Marrakesh	31.447446	-7.87704	678 m	x	x	_	x
IT0052	E6028.19	*Chalcides*	*polylepis*	Morocco	10 Km NW Marrakech	31.717131	-8.146644	360 m	x	x	x	x
IT0053	E60282	*Chalcides*	*manueli*	Morocco	Essaouira	31.515660	-9.65402	131 m	x	x	x	x
IT0054	E60283	*Chalcides*	*manueli*	Morocco	Essaouira	31.515660	-9.65402	131 m	x	x	_	_
IT0055	E60286	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.201369	-7.865269	2693 m	x	x	_	_
IT0056	E60287	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.201369	-7.865269	2693 m	x	x	_	x
IT0057	E60288	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.201369	-7.865269	2693 m	x	x	_	_
IT0058	E60289	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.201369	-7.865269	2693 m	x	x	_	x
IT0060	DB5127	*Chalcides*	*montanus*	Morocco	Tizi n'Tichka	31.26972	-7.292305	1770 m	x	x	x	x
IT0061	E25061.2	*Chalcides*	*manueli*	Morocco	Sidi Ifni	29.3865	-10.16799	15 m	x	x	x	x
IT0062	E4113.24	*Chalcides*	*manueli*	Morocco	Sidi Ifni	29.3865	-10.16799	15 m	x	x	x	x
IT0068	T486	*Chalcides*	*polylepis*	Morocco	Dar Caïd-Ouriki	31.49053	-7.91382	617m	x	x	_	x
IT0069	T487	*Chalcides*	*polylepis*	Morocco	10 Km NW Marrakesh	31.717131	-8.146644	360 m	x	x	x	x
IT0071	BEV6017	*Chalcides*	*montanus*	Morocco	Oulmes	33.425	-6.005	1188 m	x	_	_	_
IT0080	IBE-S1696	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.18379	-7.84854	2754 m	x	_	_	_
IT0081	E140372	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.255869	-7.867102	1791 m	x	_	_	_
IT0082	E25061.30	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.255869	-7.867102	1791 m	x	_	_	_
IT0083	IBE-S1695	*Chalcides*	*montanus*	Morocco	Oukaimeden	31.18379	-7.84854	2754 m	x	_	_	_
IT0085	DB24118	*Chalcides*	*montanus*	Morocco	Tinghir	31.62132	-5.56037	1566 m	x	x	x	x

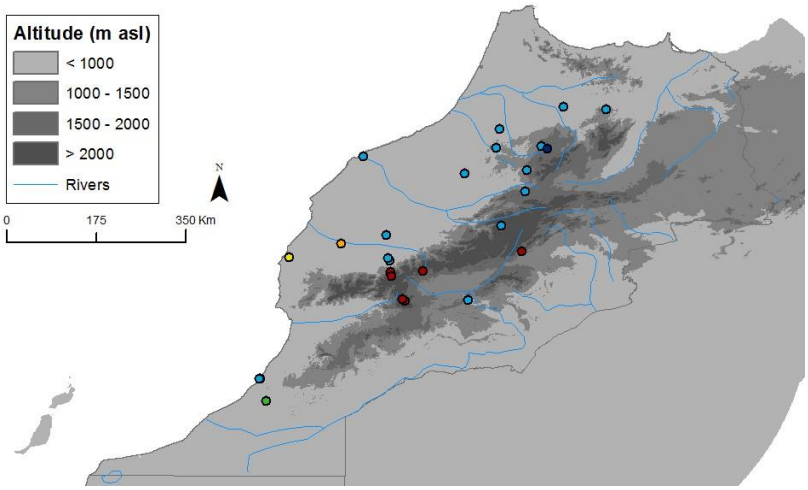

Figure 2.1 – Location of the used samples: Light blue dots - *Chalcides polylepis*; Dark blue dot – *Chalcides sp. aff. polylepis;* Yellow dots - *Chalcides manueli*; Green dots - *Chalcides mionecton*; Orange dot – *Chalcides sp.*; and Red dots - *Chalcides montanus.*

2. DNA Extraction

Using the standard saline method (Sambrook, Fritsch, & Maniatis, 1989), briefly described here, the DNA was extracted from the tissue samples preserved in 96% ethanol, (for detailed information refer to Appendix 1). A small amount of the collected sample was cut in tiny pieces and put in a 1.5 µL Eppendorf tube. 600 µL of the lysis buffer (0.5M tris, 0.1M EDTA, 2% SDS, pH 8.0) and 8 µL of proteinase *K* (25 mg/ml) were added to begin sample digestion, freeing the cellular contents. After incubation the ammonium acetate was used to precipitate and eliminate the proteins. Cold isopropanol was added to precipitate the DNA into a pellet during the next centrifugation. The final step includes the washing of the DNA pellet using cold 70% ethanol and after evaporation at room temperature, the pellet is eluted with ultrapure water.

3. DNA Amplification

Polymerase chain reactions (PCR) were used to amplify two fragments of mitochondrial DNA, 12S rRNA and Cytocrome *b* (cyt*b*), and fragments of two proteincoding nuclear DNA, the Melanocortin Receptor 1 (MC1R) and the Acetylcholinergic Receptor M4 (ACM4). The 12S and Cytb genes were chosen so the results of the study could be used in comparison to Carranza's study (2008); while ACM4 and MC1R were chosen knowing that they have been successfully used for assessing variation within other species of lacertids (Carranza et al., 2008; Barata, Carranza, & Harris, 2012). Cytb, ACM4 and MC1r are protein coding genes. The primers chosen for the amplification of each of the four gene fragments and the length of each fragment are listed in Table 2.2.

Table 2.2 – Detailed information on the primers used.

Gene	Primers (Forward/Reverse)	Primers' Sequence	Approximate fragment length
12S	12S_H	TGACTGCAGAGGGTGACGGGCGGTGTGT	320
	12S_L	AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT	
Cyt*b*	Cyt*b*1	CCATCCAACATCTCAGCATGATGAAA	340
	Cyt*b*2	CCCTCAGAATGATATTTGTCCTCA	
MC1R	MC1R_F	GGCNGCCATYGTCAAGAACCGGAACC	640
	MC1R_R	CTCCGRAAGGCRTAGATGATGGGGTCCAC	
ACM4	TgF	CAAGCCTGAGAGCAARAAGG	460
	TgR	ACYTGACTCCTGGCAATGCT	

PCR protocols and conditions are described in Tables 2.3 and 2.4 for each pair of primers. PCRs were run on a Biometra TProfessional Standard gradient Thermocycler and with each PCR run, a negative control (composed with all reagents except the DNA) was added. All PCR reagents were first prepared in a premix and 23µ was added to each reaction well, finally the DNA was added in each correspondent well.

Table 2.3 – List of PCR reagents and their volume.

Reagent	Concentration	Volume
Water	-	16.3 µL
Buffer	1 x	5 µL
Primer forward	0.5 µM	1 µL
Primer reverse	0.5 µM	1 µL
Taq	1 x	0.2 µL
Total	**-**	**23 µL**

Table 2.4 – PCR protocols for the 12S, Cyt*b*, MC1R and ACM4 primers.

Step	Function	**12S** time		#cycle	**Cyt*b*** Time		#cycl	**MC1R** Time		#cycl	**ACM4** time		#cycle
		ºC	s		ºC	es		ºC	es		ºC	s	
Initial Denaturation	Denaturation	95	1'	1	95	2'	1	92	2'	1	94	5'	1
Thermal cycling	Denaturation	95	15''	35	95	1'	35	92	1'	35	94	45''	39
	Annealing	55	15''		50	45''		55	45''		55.5	45''	
	Extension	72	40''		72	1'		72	1'		72	1'20''	
Final Extension	Extension	72	10'	1	72	7'	1	72	7'	1	72	5'	1
Hold	Hold	12	∞	1	12	∞	1	12	∞	1	12	∞	1

1µL of each PCR product was loaded on a 2% agarose gel with 1µL of GelRed Nucleic Acid Stain (10,000x in water, BIOTIUM) per 50µL of agarose solution. A reference ladder was loaded, in order to determine the size of the amplicons. The gel was placed in the electrophoresis apparatus and run at 250 volts for approximately 15 minutes and afterwards, was placed under an ultraviolet transilluminator and each gel was photographed.

4. Sequencing and Phylogenetic Analysis

Sequence Analysis

Positive PCR products were sent to a commercial sequencing facility (Beckman Coulter Genomics or Macrogen Inc.) to be sequenced. When in the facility the PCR products were run in a sequencer in which the fluorescently labeled ddNTPs (dideoxynucleotide triphosphates) fluoresce and are recorded in sequence, giving origin to the sequences. Once received, these sequences were blasted on the NCBI database to identify the most similar available sequences. After that, sequences were then manually aligned using the ClustalW software (Larkin et al., 2007) implemented in BioEdit (Hall, 1999) and reviewed one by one to make sure each sequence was correct. In the protein coding genes it was checked whether there was a STOP codon and also for the mitochondrial alignments no suspicious gaps were found.

Haplotype Network

When working with DNA alignments we should take in account if we are working with mitochondrial or nuclear DNA. Since nuDNA is double stranded and inherited both maternally and paternally (unlike mtDNA which is only inherited maternally) it can have heterozygous alleles. In order to better determine the most probable allele for heterozygous individuals, the alignments should be phased (Fouquet *et al*. 2012).

Using PHASE implemented in DNAsp v5 (Librado & Rozas, 2009), both MC1R and ACM4 alignments were phased independently and nucleotides were cut in the beginning and end of each sequence in order to set the codon position in the first site of the sequence. Different runs with different seeds were made and compared, and the best phased run was chosen to conduct the analysis. Afterwards, each of the created phased alignment was converted using FABOX (Villesen, 2007) command “DNA to haplotype collapse and converter” and a TCS input file was also created using the “Create TCS input file from fasta (fasta2tcs)” command.

Mitochondrial DNA is maternally inherited and it does not normally recombine. The absence of recombination allows sequences to be traced through one genetic line and all polymorphisms present are considered to be caused by genetic mutations. Since mtDNA has a faster mutation rate than nuDNA in mammals, mtDNA sequences are more variable. This is an advantage when studying closely related species since this taxonomic level many nuclear markers may be uninformative. However, for the construction of phylogenetic trees, the use of only mtDNA is problematic since it only represents the female lineage of the study. Therefore it is useful to analyse both mtDNA and nuclear markers, as in this study.

In TCS v1.2.1 (Clement, Posada, & Crandall, 2000) phylogenetic relationships were inferred using statistical parsimony haplotype networks implemented in the program. For each haplotype network (ACM4 and MC1R) a map with the sampled specimens location and correspondent haplotype composition were produced.

Phylogenetic Analysis

The alignments 12S and Cytb were joined in FABOX (Villesen, 2007), using the command "Fasta alignment joiner" in order to create a mitochondrial alignment. Another alignment was created using the same command for the four studied genes (12S+Cytb+MC1R+ACM4). Later, in MEGA 6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013), the protein-coding alignments (Cytb, ACM4 and MC1R) were individually opened and converted from fasta format (.fas) into mega format (.meg) and from that file, four different files for each gene were saved (an alignment including only the first codon position, a second alignment with only the second codon position, a third with only the third codon position and a fourth with both first and second codon positions).

The best fitting model for each of the new partitioned alignments and complete genes, were found using jModelTest 2.1.6 (Posada, n.d.) according to Akaike Information Criterion. The results are listed in Table 2.5.

Table 2.5 – List of Models of Evolution obtained from jModelTest 2.1.6 for the complete gene of 12S, Cytb, concatenated 12S+Cytb, MC1R, ACM4 and concatenated 12S+Cytb+MC1R+ACM4, and 1st, 2nd, 3rd and 1st + 2nd position for the Cytb, MC1R and ACM4 genes.

	Partition Name	Model of Evolution				
		Complete Gene	**1st Position**	**2nd Position**	**3rd Position**	**1st + 2nd Position**
12S	**12S**	HKY+G	-----	-----	-----	-----
CYTB	**Cytb**	GTR+G	K80+I	HKY	HKY+G	HKY+I
12S+CYTB	**Mitochondrial**	HKY+I+G	-----	-----	-----	-----
MC1R	**MC1R**	GTR+G	GTR+I	K80	F81	GTR+I
ACM4	**ACM4**	GTR	F81	F81	K80	F81
12S+CYTB+MC1R+ACM4	**Total**	HKY+I+G	-----	-----	-----	-----

PartitionFinder (Lanfar, Calcott, Ho, & Guindon, 2012; Lanfear, Calcott, Kainer, Mayer, & Stamatakis, 2014) is a program used for selecting the best-fit partitioning schemes and models of molecular evolution for the nucleotide alignments. A previously prepared alignment, with the pre-defined datablocks (such as the 1st, 2nd and 3rd codon positions of the protein coding genes) is put to run in the software and an output with the best partitions and the models for each is given (see Table 2.6).

Table 2.6 – Models of evolution for each of the partitions PartitionFinder gave for the 12S+cytb+MC1R+ACM4 partitioned alignment.

Partition	Model of Evolution
12S	HKY+I
MC1R_pos2 + cytb_pos1	K80+I+G
ACM4_pos3 + cytb_pos2	K80+I
Cytb_pos3	HKY+G
MC1R_pos1	GTR+G
MC1R_pos3	F81+I
ACM4_pos1	GTR+I
ACM4_pos2	F81

MrBayes 3.2.0 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) was used for the creation of the phylogenetics trees using the models given my jModelTest and PartitionFinder, the parameters for each of the used models are in Table 2.7. It was run 10000000 generations win a print and sample frequency of 100, there was used four chains in the run. A correction of 40% was used.

Table 2.7 – List of the models given by jModelTest for the created alignments.

Model	Parameters
HKY	lset applyto = (all) nst=2 rates=equal; prset applyto=(all) statefreqpr=dirichlet(1,1,1,1);
HKY+I	lset applyto = (all) nst=2 rates=propinv; prset applyto=(all) statefreqpr=dirichlet(1,1,1,1);
HKY+G	lset applyto = (all) nst=2 rates=gamma; prset applyto=(all) statefreqpr=dirichlet(1,1,1,1);
HKY+I+G	lset applyto = (all) nst=2 rates=invgamma; prset applyto=(all) statefreqpr=dirichlet(1,1,1,1);
GTR	lset applyto = (all) nst=6 rates=equal; prset applyto=(all) statefreqpr=dirichlet(1,1,1,1);
GTR+I	lset applyto = (all) nst=6 rates=propinv; prset applyto=(all) statefreqpr=dirichlet(1,1,1,1);
GTR+G	lset applyto = (all) nst=6 rates=gamma; prset applyto=(all) statefreqpr=dirichlet(1,1,1,1);
F81	lset applyto = (all) nst=1 rates=equal; prset applyto=(all) statefreqpr=dirichlet(1,1,1,1);
F81+I	lset applyto = (all) nst=1 rates=propinv; prset applyto=(all) statefreqpr=dirichlet(1,1,1,1);
K80	lset applyto = (all) nst=2 rates=equal; prset applyto=(all) statefreqpr=fixed(equal);
K80+I	lset applyto = (all) nst=2 rates=propinv; prset applyto=(all) statefreqpr=fixed(equal);
K80+I+G	lset applyto = (all) nst=2 rates=invgamma; prset applyto=(all) statefreqpr=fixed(equal);

MLTreefinder (Jobb, 2011) estimates a maximum likelihood phylogenetic tree according to models of sequence evolution given by the program as well and so, phylogenetic trees of the following alignments were estimated using the program: 12S, 12S+Cytb, 12S+Cytb+MC1R+ACM4, ACM4, CYTB and MC1R. Bootstraps with 1000 replicates were run in the program (models in Table 2.8).

Table 2.8 – Models of evolution by MLTreeFinder for the study genes.

Alignment	Model
12S	{HKY[Optimum,Empirical]:G[Optimum]:5}
12S_Cyt*b*_MC1R_ACM4	{HKY[Optimum,Empirical]:G[Optimum]:5, HKY[{3,1,1,1,1,3},Empirical], HKY[{3,1,1,1,1,3},Empirical], HKY[{3,1,1,1,1,3},Empirical], HKY[Optimum,Empirical]:G[Optimum]:5, HKY[{3,1,1,1,1,3},Empirical]:G[Optimum]:5, HKY[{3,1,1,1,1,3},Empirical], HKY[{3,1,1,1,1,3},Empirical], HKY[{3,1,1,1,1,3},Empirical], HKY[{3,1,1,1,1,3},Empirical]}
12S_Cyt*b*	{HKY[Optimum,Empirical]:G[Optimum]:5, HKY[{3,1,1,1,1,3},Empirical], HKY[{3,1,1,1,1,3},Empirical], HKY[{3,1,1,1,1,3},Empirical]}
ACM4	{HKY[{3,1,1,1,1,3},Empirical], HKY[{3,1,1,1,1,3},Empirical], HKY[{3,1,1,1,1,3},Empirical]}
Cyt*b*	{HKY[{3,1,1,1,1,3},Empirical], HKY[{3,1,1,1,1,3},Empirical], HKY[{3,1,1,1,1,3},Empirical]}
MC1R	{HKY[{3,1,1,1,1,3},Empirical], HKY[{3,1,1,1,1,3},Empirical], HKY[{3,1,1,1,1,3},Empirical]}

Pairwise Differences

In order to obtain the genetic distances between and within groups, the 12S alignment (the most complete of all) was opened in MEGA and converted into mega format. The alignment was divided into the number of groups given by the phylogenetic tree and the distance between and within groups was calculated. The parameters used in each analysis are resumed in Table 2.9.

Table 2.9 – Parameters used for the calculation of genetic distance between and within groups.

	Distance Within and Between Groups
Estimate Variance	
Variance Estimation Method	None
Substitution Model	
Substitutions Type	Nucleotide
Model / Method	p-distance
Substitutions to include	d: Transitions + Transversions
Rates and Patterns	
Rates among Sites	Uniform rates
Pattern among Lineages	Same (Homogeneous)
Data Subset to Use	
Gaps / Missing Data Treatment	Complete deletion

CHAPTER 3: RESULTS

1. Phylogenetic Tree

In the estimate of relationships derived from the Bayesian analysis (Figure 3.1), the samples were distributed into 12 distinct lineages: firstly, in the out-group, are the two specimens of *C. mionecton* from Guelmine; the first lineage separating within the ingroup is formed by specimens from Oukaimeden, Oulmes and 10 km NW Marrakesh, from which, the samples from Marrakesh are *C. polylepis*, while the others are all *C. montanus*; in the second lineage, there are samples of *C. polylepis* from 18 km Marrakesh and Dar Caïd-Ouriki; three specimens of *C. manueli* from Essaouira form the third lineage; the forth lineage has two specimens, one *Chalcides sp.* from Sidi Chiker and one *C. polylepis* from Marrakesh; the fifth lineage only has one specimen, *C. polylepis* from Azemmour; there is only one *C. montanus* from Tinghir in the sixth lineage while the seventh lineage has four *C. manueli* from Sidi Ifni and one specimen of *C. sp. aff. polylepis* from Mischliffen; lineage eight is formed only by a *C. polylepis* from Sidi Ifni; in the ninth lineage we can find eight specimens of *C. montanus* from Jbel Siroua and one from Tizin Tichka; the four *C. polylepis* from the tenth lineage are all from different north Moroccan locations, Azrou, Hamryia, Khémisset and Taza; in the last lineage there are only *C. polylepis* from the following localities Anergui, Azrou, Medium Atlas, Oulmes and Sidi Azigza.

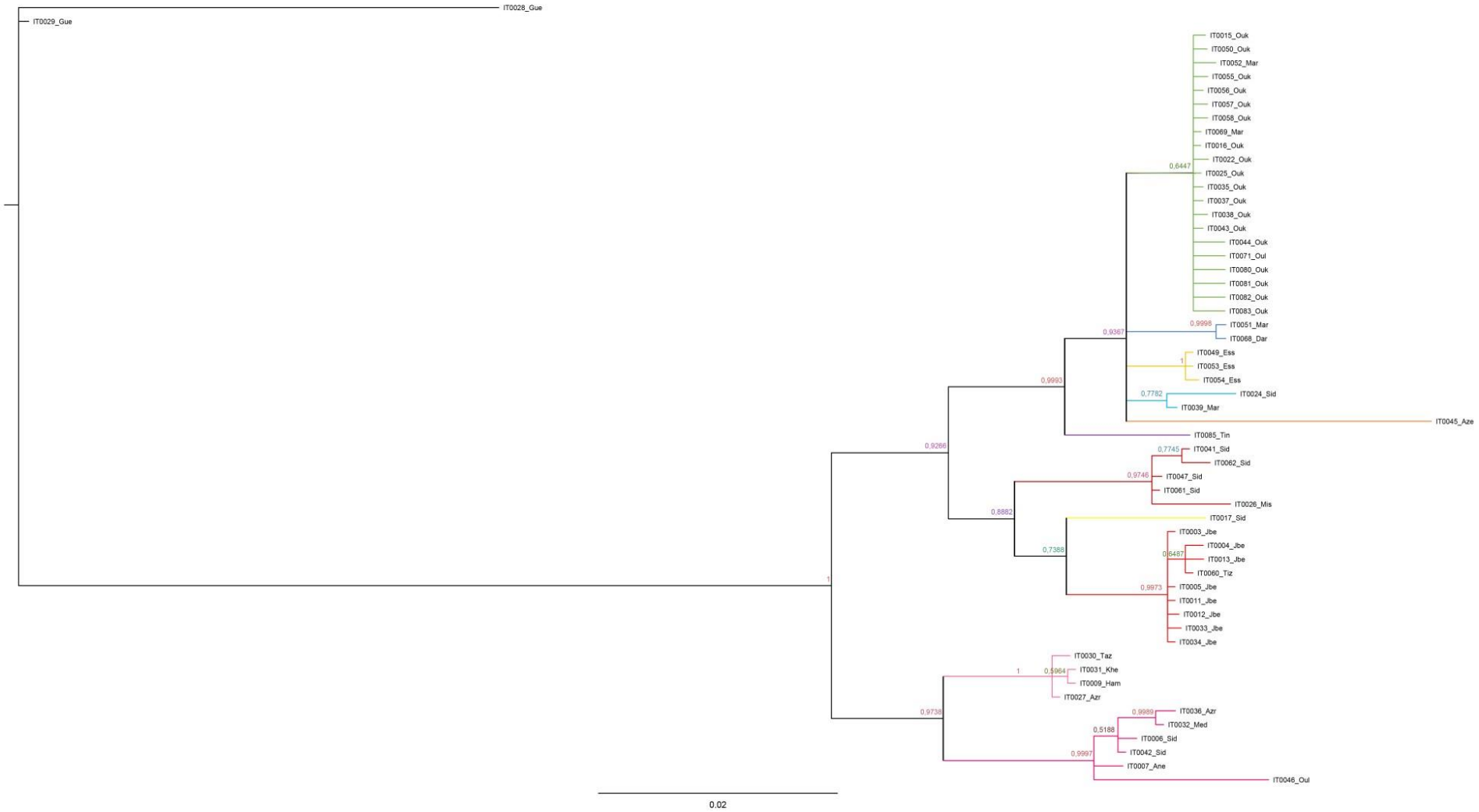

Figure 3.1 – Estimate of relationships derived from a Bayesian analysis of the four concatenated and partitioned genes (12S + Cytb + MC1R + ACM4). Values beside nodes correspond to Posterior Probability values. The lineages are identified by colours: Lineage 1 – **Green**; Lineage 2 – **Dark Blue**; Lineage 3 – **Dark Yellow**; Lineage 4 – Light Blue; Lineage 5 – **Orange**; Lineage 6 – **Purple**; Lineage 7 – **Dark Red**; Lineage 8 – **Light Yellow**; Lineage 9 – **Light Red**; Lineage 10 – **Light Pink**; Lineage 11 – **Dark Pink**.

2. Haplotype Networks

ACM4 Haplotype Network

The resulting haplotype network for the ACM4 gene consists of 29 haplotypes separated by a maximum of four mutational steps. However, it should be noted that the sample size is very restricted for some lineages.

The most common haplotype (A14) has predominantly *Chalcides polylepis* from northern Morocco, with populations from lineage 10: Azrou, Hamriyah and Khémisset; and lineage 11: Anergui, Medium Atlas, Sidi Azigza and Sidi Yahya. Other specimens with this haplotype include individuals from the populations from Sidi Chiker and Marrakesh, from lineage 4 and a single sample of *C. polylepis* from Sidi Ifni (lineage 8).

The second most common haplotype is the A2 and presents populations from Tizi n'Tichka, Jbel Siroua, Sidi Ifni, Essaouira and Oukaimeden. This seems to unite *Chalcides manueli* and *Chalcides montanus* as closely related. The haplotype network can be seen in more detail in Figure 3.2.

It is possible to see that this nuclear marker does not show a clear separation between the three species.

ACM4 Haplotype Map

Localities from the north of Morocco seem very similar with each other, being differentiated into two different lineages even though specimens from these areas include a total of four different haplotypes. Regarding the other populations, Oukaimeden presents the locality with the highest number of haplotypes, 8, four of which represent half of the sampled population.

Jbel Siroua shares one haplotype with a population of Tizin n'Tichka, having between both locations, the only samples from lineage 9. Marrakesh and Sidi Chiker share one of the two haplotypes present in each location, even though this shared haplotype (A14) is also present in populations from Sidi Ifni, Sidi Azigza, Anergui, Oulmes, Khémisset, Medium Atlas, Azrou and Hamriyah.

Three of the five haplotypes present in Sidi Ifni are unique to that location, the other two, A2 and A14 are also present in locations such as Jbel Siroua, Tizi n'Tichka, Oukaimeden and Essaouira, and Sidi Chiker, Marrakesh, and some of the northern locations, respectively. The haplotype map can be seen in more detail in Figure 3.3.

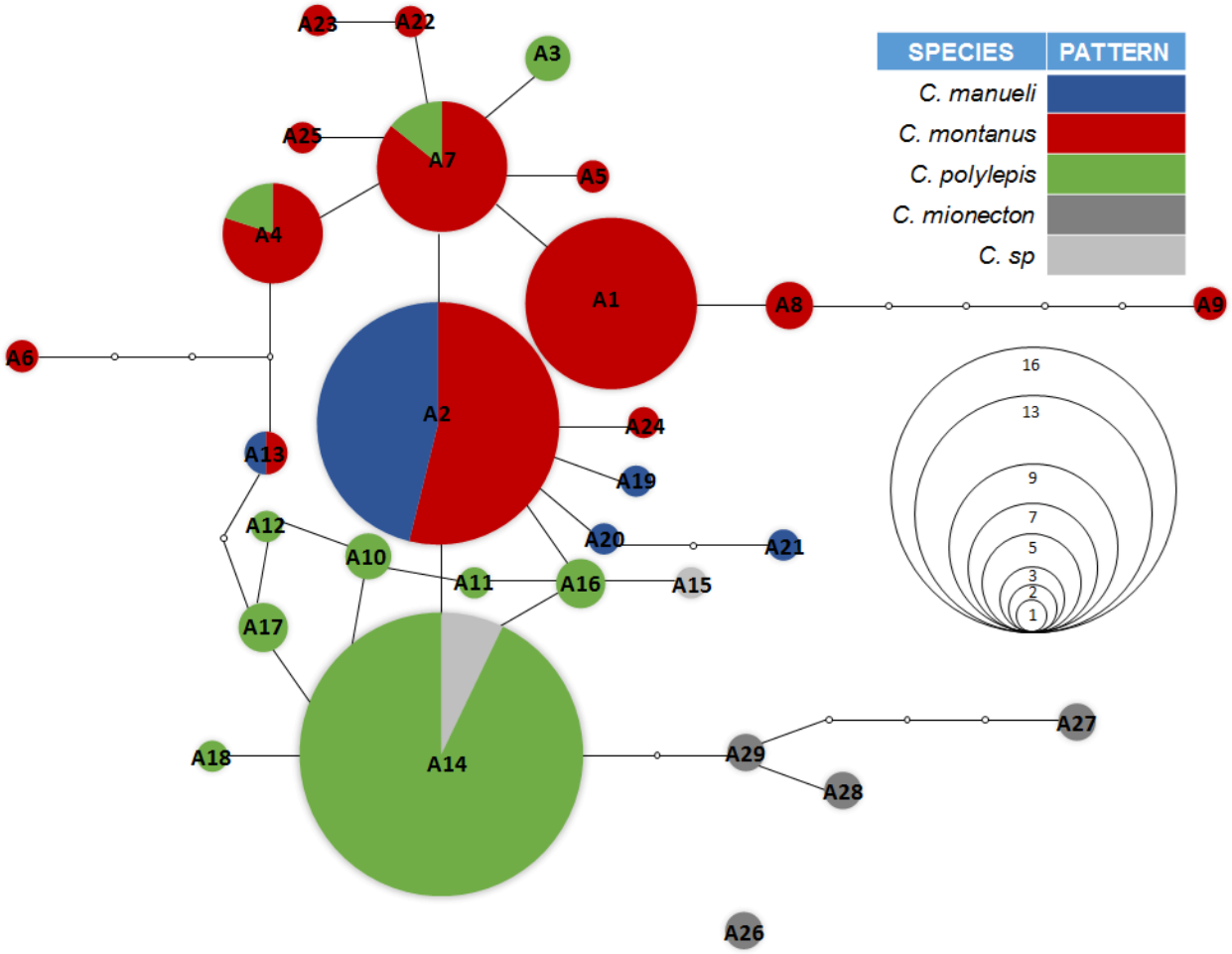

Figure 3.2 – Haplotype Network for the ACM4 gene.

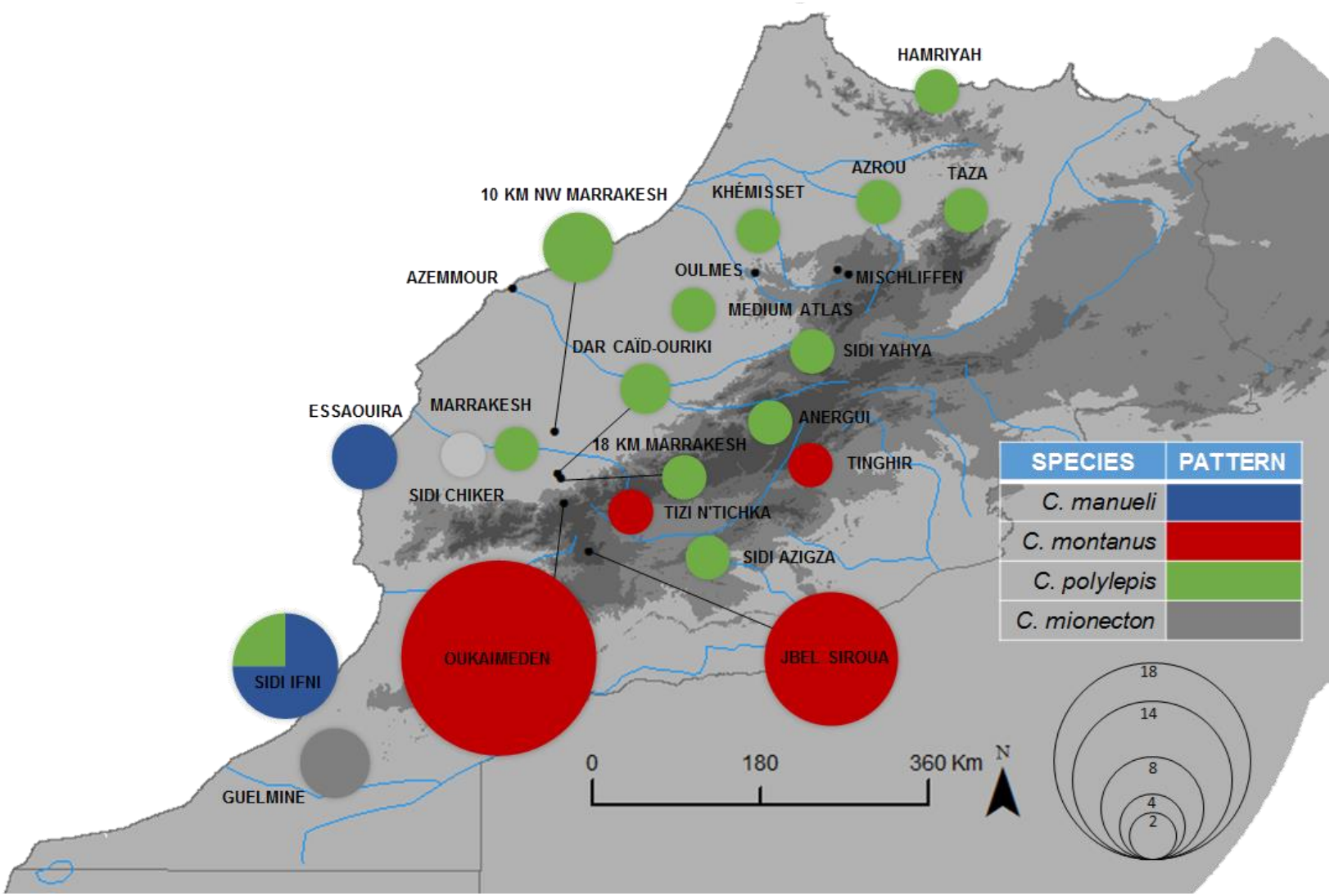

Figure 3.3 – Map of the Haplotypes for the ACM4 gene.

MC1R Haplotype Network

The resulting haplotype network for the MC1R gene consists of a total of 40 haplotypes separated by a maximum of nine mutational steps. Again however, the sample size is very restricted.

The commonest haplotype of this network (M3) occupies a central position within the network and is predominantly comprised of *Chalcides montanus* from lineage 1: Oukaimeden and 10 km NW Marrakesh; and lineage 9: Jbel Siroua and Tizi n'Tichka. All but one haplotypes from Essaouira samples cluster together, and the M13, M14 and M16 haplotypes from Essaouira are also similar to haplotypes from Sidi Ifni, having one mutational step between them, and between samples from Oukaimeden.

The second most common haplotype is the M2 and presents populations from Oukaimeden, 18 km Marrakesh and Dar Caïd-Ouriki. A group of haplotypes (M1, M7, M9 and M25) are also very similar to the first one and also is present in populations from Oukaimeden (M1, M7 and M9), Sidi-Chiker (M1) and Tizi n'Tichka (M25). The haplotype network can be seen in more detail in Figure 3.4.

Just as before, it is possible to see that this nuclear marker does not show a clear separation between the three species.

MC1R Haplotype Map

As happened for the ACM4 gene, again localities from the north of Morocco seem very similar with each other, being differentiated into two different lineages. In this case, instead of having a total of four different haplotypes, for the MC1R gene, there are eleven haplotypes present in the area. Regarding the other populations, Oukaimeden presents the locality with the largest number of haplotypes, with nine haplotypes. The sample from 10 km NW Marrakesh shares the same haplotype as one of Oukaimedens' samples.

Again, Jbel Siroua shares one haplotype with a population of Tizin n'Tichka, having between both locations, the only samples from lineage 9. Contrary to what happen in the ACM4 haplotype map, the populations of Marrakesh and Sidi-Chiker do not share any of the two haplotypes present in each location between them. However, one of the haplotypes present in Sidi Chiker (M1) is also present in Oukaimeden and one from Marrakesh (M19) is also present in the population of Anergui. The haplotype map can be seen in more detail in Figure 3.5.

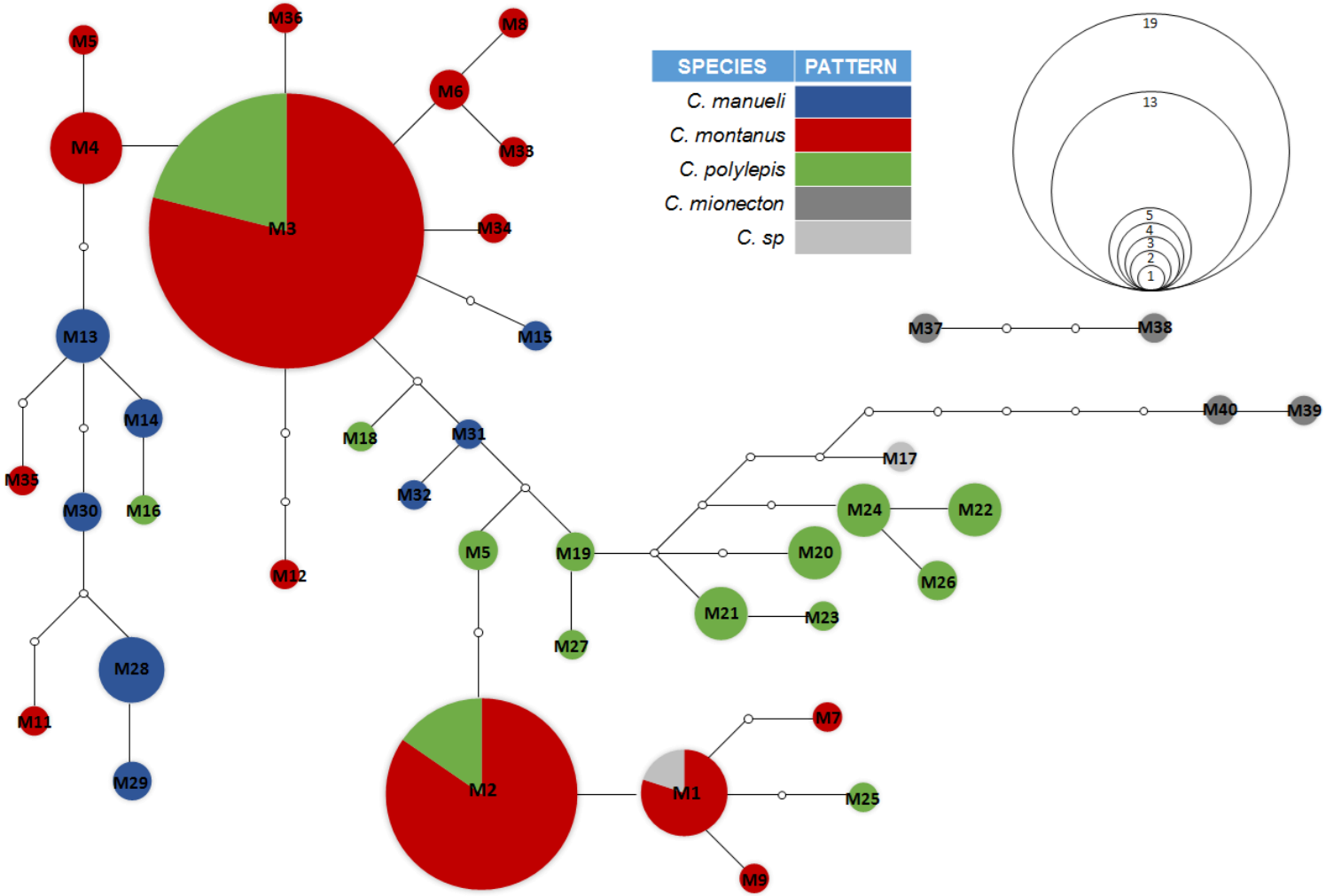

Figure 3.4 – Haplotype Network for the MC1R gene.

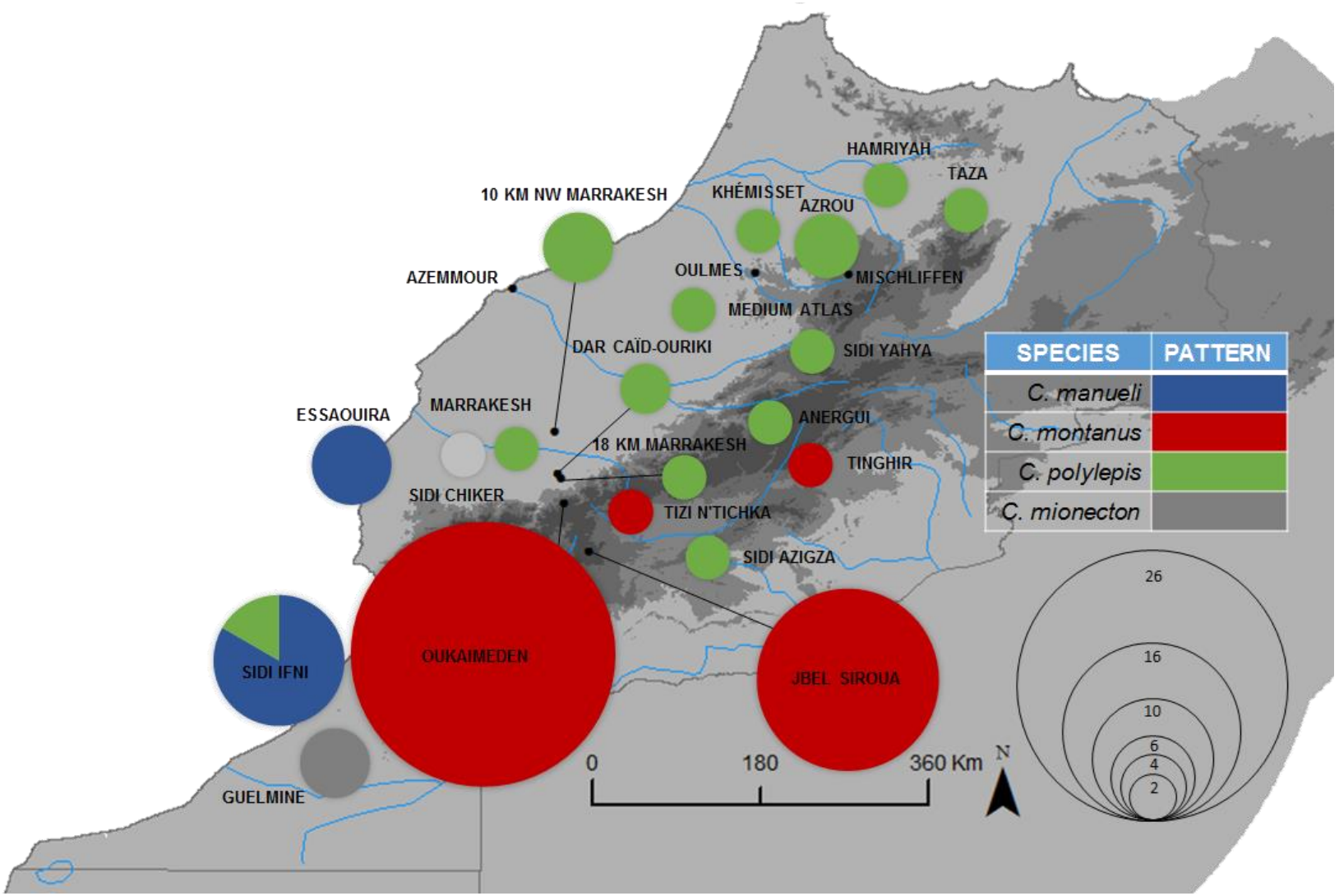

Figure 3.5 – Map of the Haplotypes for the MC1R gene.

All of the five haplotypes from Sidi Ifni are unique to that location, which also happens for the haplotypes present in Guelmine, belonging to the out-group. In Essaouira only populations from lineage 3 are present, sharing four haplotypes between them.

Dar Caïd-Ouriki and 18 km Marrakesh share the same haplotypes, one of which shared with the population of Tinghir. The other haplotype present in Tinghir is present only in Essaouira.

3. Genetic Distances

In table 4.1 the Pairwise uncorrected differences within and between groups are represented. The groups were defined according to the lineages provided by the Bayesian Phylogenetic Tree (Figure 3.1). The genetic divergence (uncorrected p-distance) obtained with the 12S fragment varies between 0% and 0.9%. The most diverse groups are number 4 and 11. Group 4 has one sample from Marrakesh and another from Sidi Chiker while group 11 has samples from Anergui, Azrou, Medium Atlas, Oulmes and Sidi Yahya. Group 10 also has one sample from each locality (Azrou, Hamriyah, Khémisset and Taza) however it presents a p-distance of 0% between individuals. Group 1, 7 and 9 also have samples from more than one locality, however the p-distances for these groups are 0.0% for 1 and 9 and 0.1% for group 7.

It was not possible to calculate the within group variation for groups 5, 6 and 8 since they are constituted only by one sample.

The major genetic distances appear between groups Out1 (*C. mionecton*) and Out2 (*C. polylepis*) (11.9%). The lowest genetic distance occurs between groups 2 and 3, and 7 and 9 (1.3%).

Table 3.1 – Pairwise uncorrected distances (p-distances) within and between groups.

Within Groups		*Between Groups*											
Group	p-distance	Group	1	2	3	4	5	6	7	8	9	10	11
1	0.000	1											
2	0.000	2	0.016										
3	0.000	3	0.016	0.013									
4	0.009	4	0.014	0.017	0.017								
5	n/c	5	0.019	0.025	0.025	0.020							
6	n/c	6	0.028	0.019	0.025	0.030	0.031						
7	0.001	7	0.041	0.038	0.038	0.037	0.032	0.032					
8	n/c	8	0.063	0.060	0.060	0.055	0.053	0.050	0.026				
9	0.000	9	0.047	0.044	0.044	0.042	0.038	0.038	0.013	0.025			
10	0.000	10	0.050	0.047	0.044	0.044	0.044	0.041	0.035	0.044	0.034		
11	0.009	11	0.060	0.057	0.057	0.054	0.054	0.051	0.045	0.061	0.044	0.028	

CHAPTER 4: DISCUSSION

Species' delimitation is an important and controversial area in evolutionary biology. In recent years, the methods available for species' delimitation have been increasing even though recent investigations only use a handful of the available methodologies (Carstens, Pelletier, Reid, & Satler, 2013).

Chalcides' phylogeny has been an object of study for more than 250 years, since Linneaus described *Chalcides chalcides* in 1758. The use of morphologic traits has been very common until very recently, when genetical analysis started to become more common. Even though individuals of this genus present a very similar morphology, and particularly small to reduced limbs and elongated body, their morphological traits are still much in use in their identification in the field. The samples used in this study were labelled in the field by their collectors, and detailed photographs taken. In particular, *C. manueli* is easily identified by its colour pattern. Given this we can assume that all of the specimens were correctly identified.

Regarding the current taxonomy we can begin with comparing alternative hypotheses with our estimate of phylogeny.

- **Null Hypothesis (H_0)**: the current taxonomy is correct;
- **First Hypothesis (H_1)**: the current taxonomy is correct but there is mtDNA introgression between *Chalcides montanus* and *C. polylepis*; - **Second Hypothesis (H_2)**: the current taxonomy dos not reflect the current relationships between the three species.

According to the **Null Hypothesis** (H_0), **if** the current taxonomy is correct *Chalcides manueli*, *C. montanus* and *C. polylepis* are three differentiated species, albeit morphologically similar. With the inclusion of new samples in the analysis, which Carranza *et al.* (2008) did not have, it is clear that none of the currently described species form monophyletic units. The previous analysis of Carranza et al. (2008) did not identify this due to the more limited sampling. In this earlier analysis, *C. manueli* was monophyletic, but a single specimen of *C. montanus* was embedded within *C. polylepis*. One possible explanation for this, since the analysis was based solely on mtDNA, could have been mtDNA introgression.

Carranza *et al.* (2008) suggested that *C. montanus* might have received mitochondrial DNA from *C. polylepis* through introgression, probably involving a male *C. montanus* and a female *C. polylepis*. This is the **First Hypothesis** (**H_1**). However, the analysis performed for this thesis do not support this hypothesis. Not only are all three species paraphyletic with our enhanced sampling, but the network analyses of both nuclear markers also do not indicate that the three currently proposed species are

monophyletic since the nuclear markers contradict the current taxonomy. And so this is not the result of mtDNA introgression which means the taxonomy should change. But to what?

The simplest approach is that this is only one species even though a quite divergent one. What does this mean for morphology?

Chalcides is a highly variable genus with high morphological plasticity. In a recent study it was observed that the number of digits varies in a very complex manner, with *C. mionecton* individuals with five digits being more common in northern Morocco, and that this character is polymorphic in some populations (Brown et al. 2012). This is concordant with the pattern of body dimension variation but it not a useful character for determining species delimitation (Brown, et al. 2012). The same authors also found that some northern populations are morphologically similar to others from southern Morocco even though these fall into discrete genetic clusters (Brown et al., 2012).

The morphological variability within the species may be explained by environmental factors associated with the presence of predators. Different individuals of a population may be adapted to somewhat different environments according to three different conditions: when dividing the population into sets of individuals, individuals of a determined set survive and reproduce better than those of another set in a certain environment A, while in an environment B, the other set of individuals might be more adapted; the difference between this sets of population has a genetic portion; and the presence of a distributional or mating preference (Valen, 1965).

For instance, *C. montanus* and *C. polylepis* have a very similar, if not the same, dorsal color pattern (see Figure 4.1), differing primarily in their body size. *C. montanus*' smaller body size than *C. polylepis* can be explained by the altitudes in which the first inhabits. Species living in higher altitude habitats tend to have smaller body sizes than their lower altitude living counterparts. The mean value of a given trait in a population is determined by the interaction of genetic factors with environmental influences and is considered to be a form of adaptation to the local environmental conditions (Craig Stillwell & Fox, 2009). Species adaptation to local environmental conditions and selective agents is represented by differences among different populations. However it is still not very well understood whether the degree of variation is due to adaptation or plasticity (Stillwell & Fox, 2009).

Figure 4.1 – *Chalcides montanus* (A) (from: https://www.flickr.com/photos/82470440@N06/8404230760/in/photolistakXadx-6X5wsx-dNDT1m) and *Chalcides polylepis* (B) similar dorsal patterns (picture by Daniele Salvi, Morocco 2013).

On the other hand, *C. manueli* and *C. montanus* have similar size however the first does not have a dorsal colour pattern, contrasting with the second (Figure 4.2). *C. manueli* specimens live in coastal areas, often near beaches and rivers, and so, in sandy environments. The lack of a dorsal colour pattern might be explained by the presence of morph specific predators (Farallo *et al.* 2012). For example, in the event of the presence of a predator, an individual with dorsal colour pattern would have been more easily caught than another lacking this pattern. Since *C. manueli* specimens are yellowishbrown, it is relatively easy for them to hide or to pass unnoticed when living in a sandy habitat, since the individuals' colour resembles the sand. Colour polymorphism is the most striking example on phenotypic biodiversity and has been documented in a variety of different taxa (Hoagland, 1977; Sandoval & Crespi, 2008; Sinervo, Bleay, & Adamopoulou, 2001). It raises several questions such as why have multiple phenotypes within a single species evolved and how are they maintained. Heterogeneous habitats and substrates are one of the factors contributing to colour polymorphisms (Byers, 1990; Hoekstra, Drumm, & Nachman, 2004; Rosenblum, 2006; Vignieri, Larson, & Hoekstra, 2010).

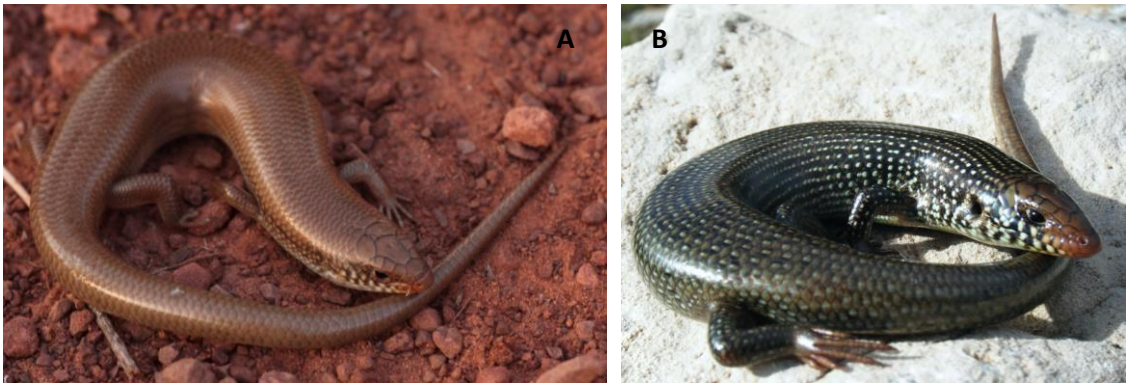

Figure 4.2 - *Chalcides manueli* (A) (from: https://www.flickr.com/photos/alberto_herpetology/14016912974/in/photolistnmCkTm-njLg6U-nmiBMv-njxtqu) and *Chalcides montanus* (B) similar dorsal patterns (from: https://www.flickr.com/photos/82470440@N06/8404230760/in/photolist-akXadx-6X5wsx-dNDT1m).

There are three main hypothesis for the conservation of colour variation among populations:

- Thermoregulation which is supported in a varied number of studies (Forsman, Ringblom, Civantos, & Ahnesjö, 2002; Goulson, 1994; Johnston, 1996);
- Crypsis (A. B. Bond, 2007) is the capability some species have to avoid observation or detection by others. This may be expressed by camouflage, nocturnality, subterranean lifestyle, transparency and mimicry, among others. This hypothesis is based on the assumption that cryptic coloration prevails in a given area as a result of foraging success of cryptic predators;
- Cryptic coloration evolves in areas with different substrates, reducing effectively the potential predation by visual observation of the predator (A. Bond & Kamil, 2006; Vignieri et al., 2010).

Given the habitat of *C. manueli*, the evolutionary trend towards a uniform dorsal colouration in populations living in sandy areas seems like a reasonable hypothesis. Therefore it is relatively easy to explain how the morphological differences between forms could have arisen from a morphologically plastic ancestor. This in turn can be used to support our proposal of the recognition of a single variable species, *C. polylepis*.

CHAPTER 5: FINAL CONCLUSIONS

The presence of new samples in the study resulted in a more complete phylogenetic tree which is not compatible with the current *Chalcides* taxonomy of the three study species (*C. manueli*, *C. montanus* and *C. polylepis*). Also the results refusing both mtDNA and nuDNA genes does not support the hypothesis of mtDNA introgression between a *C. montanus* male and a *C. polylepis* female. So it seems that the current taxonomy should be changed and that the simplest explanation would be the acceptance of a single, albeit morphologically variable species, *Chalcides polylepis*, since from the three species in study, *C. polylepis*, was the first described, in 1890 by Boulenger. This would then be a morphological diverse species, presenting variation in size as well as dorsal colour pattern.

In future studies, a more detailed morphological study should be taken into account and the sampling effort should be greatly increased. *Chalcides* are burrowing species, and escape by digging into the soil and plant roots. This factor allied to the fact that they are difficult to detect, makes obtaining specimens much more complicated. It would also be valuable to perform detailed assessments on the area of possible sympatry between the three forms of *Chalcides polylepis*. Furthermore, since based on our assessment the morphological characters used in species delimitation within *Chalcides* are highly homoplastic, it would be valuable to re-examine the taxonomy of various other lineages within this group.

REFERENCES

Avise, J. C. (2009). Phylogeography: retrospect and prospect. *Journal of Biogeography*, *36*(1), 3–15. doi:10.1111/j.1365-2699.2008.02032.x

Barata, M., Carranza, S., & Harris, D. J. (2012). Extreme genetic diversity in the lizard *Atlantolacerta andreanskyi* (Werner, 1929): A montane cryptic species complex. *BMC Evolutionary Biology*, *12*(1), 167. doi:10.1186/1471-2148-12-167

Bond, A. B. (2007). The Evolution of Color Polymorphism: Crypticity, Searching Images, and Apostatic Selection. *Annual Review of Ecology, Evolution, and Systematics*, *38*(1), 489–514. doi:10.1146/annurev.ecolsys.38.091206.095728

Bond, A., & Kamil, A. (2006). Spatial heterogeneity, predator cognition, and the evolution of color polymorphism in virtual prey. *Proceedings of the National Academy of Sciences*, *103*(9), 3214–9. doi:10.1073/pnas.0509963103

Bons, J., & Geniez, P. (1996). *Amphibians & Reptiles of Morocco (Including Western Sahara) Biogeographic Atlas*. (A. Montori & V. Roca, Eds.) (p. 320 pp.). Barcelona: Asociación Herpetológica Española.

Brown, R. P., Tejangkura, T., El Mouden, E. H., Ait Baamrane, M. a, & Znari, M. (2012). Species delimitation and digit number in a North African skink. *Ecology and Evolution*, *2*(12), 2962–73. doi:10.1002/ece3.326

Byers, B. A. (1990). Shell colour polymorphism associated with substrate colour in the intertidal snail *Littorina saxatilis* Olivi (Prosobranchia: Littorinidae). *Biological Journal of the Linnean Society*, *40*, 3–10.

Caputo, V. (2004). The cranial osteology and dentition in the scincid lizards of the genus *Chalcides* (Reptilia, Scincidae). *Italian Journal of Zoology*, *71*(sup2), 35–45. Retrieved from http://www.tandfonline.com/doi/abs/10.1080/11250000409356604

Caputo, V., Lanza, B., & Palmieri, R. (1995). Body elongation and limb reduction in the genus *Chalcides* Laurenti 1768 (Squamata Scincidae): a comparative study. *Tropical Zoology*, *8*(1), 95–152. Retrieved from http://dx.doi.org/10.1080/03946975.1995.10539275

Caputo, V., & Mellado, J. (1992). A new species of *Chalcides* (Reptilia: Scincidae) from northeastern Morocco. *Bolletino Di Zoologia*, *59*(3), 335–342. doi:10.1080/11250009209386690

Carranza, S., Arnold, E. N., Geniez, P., Roca, J., & Mateo, J. a. (2008). Radiation, multiple dispersal and parallelism in the skinks, *Chalcides* and *Sphenops* (Squamata: Scincidae), with comments on *Scincus* and *Scincopus* and the age of the Sahara Desert. *Molecular Phylogenetics and Evolution*, *46*(3), 1071–1094. doi:10.1016/j.ympev.2007.11.018

Carstens, B. C., Pelletier, T. a, Reid, N. M., & Satler, J. D. (2013). How to fail at species delimitation. *Molecular Ecology*, *22*(17), 4369–83. doi:10.1111/mec.12413

Clement, M., Posada, D., & Crandall, K. (2000). TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, *9*(10), 1657–1660.

Crottini, A., Dordel, J., Köhler, J., Glaw, F., Schmitz, A., & Vences, M. (2009). A multilocus phylogeny of Malagasy scincid lizards elucidates the relationships of the fossorial genera *Androngo* and *Cryptoscincus*. *Molecular Phylogenetics and Evolution*, *53*(1), 345–350. doi:10.1016/j.ympev.2009.05.024

Forsman, A., Ringblom, K., Civantos, E., & Ahnesjö, J. (2002). Coevolution of color pattern and thermoregulatory behavior in polymorphic pygmy grasshoppers *Tetrix undulata*. *Evolution; International Journal of Organic Evolution*, *56*(2), 349–60. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11926503

Giovannotti, M., Cerioni, P. N., Kalboussi, M., Aprea, G., & Caputo, V. (2007). Phylogeographic inferences from the mtDNA variation of the three-toed skink, *Chalcides chalcides* (Reptilia: Scincidae). *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution*, *308*(3), 297–307. doi:10.1002/jez.b

Glasby, C. G., Ross, G. J. B., & Beesley, P. L. (1993). *Fauna of Australia Volume 2A Amphibia and Reptilia*. (C. G. Glasby, G. J. B. Ross, & P. L. Beesley, Eds.) (p. viii + 439 pages). AGPS Canberra.

Goulson, D. (1994). Determination of larval melanization in the moth, *Mamestra brassicae*, and the role of melanin in thermoregulation. *Heredity*, *3*, 471–479. Retrieved from http://www.nature.com/hdy/journal/v73/n5/abs/hdy1994145a.html

Greenbaum, E., Campbell, A. C., & Raxworthy, C. J. (2006). A Revision of sub-Saharan *Chalcides* (SQUAMATA : SCINCIDAE), with redescriptions of two east African species. *Herpetologica*, *62*(1), 71–89.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, *41*, 95–98.

Hoagland, K. E. (1977). A gastropod color polymorphism: one adaptive strategy of phenotypic variation. *Biological Bulletin*, *152*, 360–372.

Hoekstra, H., Drumm, K., & Nachman, M. (2004). Ecological genetics of adaptive color polymorphism in pocket mice: geographic variation in selected and neutral genes. *Evolution*, *58*(6), 1329–41. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15266981

Hoser, R. T., Road, P., & Orchards, P. (2012). A four-way division of the skink genus *Chalcides* Laurenti , 1768 (Squamata : Sauria : Scincidae)., *5698*(June), 27–30.

Huelsenbeck, J. P., & Ronquist. (2001). MRBAYES: Bayesian inference of phylogeny. *Bioinformatics*, *17*, 754–755.

Jobb, G. (2011). *TREEFINDER version of March 2011. Munich, Germany. Distributed by the author at www.treefinder.de .*

Johnston, G. (1996). Genetic and seasonal variation in body colour of the Australian death, adder, *Acanthophis antarcticus* (Squamata: Elapidae). *Journal of Zoology*, *239*, 187–196. Retrieved from http://onlinelibrary.wiley.com/doi/10.1111/j.14697998.1996.tb05446.x/full

Keppel, G., Van Niel, K. P., Wardell-Johnson, G. W., Yates, C. J., Byrne, M., Mucina, L., … Franklin, S. E. (2012). Refugia: identifying and understanding safe havens for biodiversity under climate change. *Global Ecology and Biogeography*, *21*(4), 393–404. doi:10.1111/j.1466-8238.2011.00686.x

Lanfar, R., Calcott, B., Ho, S. Y. W., & Guindon, S. (2012). PartitionFinder: Combined Selection of Partitioning Schemes and Substitution Models for Phylogenetic Analyses. *Molecular Biology and Evolution*, *29*(6), 1695–1701.

Lanfear, R., Calcott, B., Kainer, D., Mayer, C., & Stamatakis, A. (2014). Selecting optimalpartitioning schemes for phylogenomic datasets. *BMC Evolutionary Biology2*, *14*(82).

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Higgins, D. G. (2007). ClustalW and ClustalX version 2. *Bioinformatics*, *23*(21), 2947–2948.

Librado, P., & Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, *25*(11), 1451–1452.

Médail, F., & Quézel, P. (1999). Biodiversity Hotspots in the Mediterranean Basin : Setting Global Conservation Priorities, *13*(6), 1510–1513.

Myers, N., Mittermeier, R. a, Mittermeier, C. G., da Fonseca, G. a, & Kent, J. (2000). Biodiversity hotspots for conservation priorities. *Nature*, *403*(6772), 853–8. doi:10.1038/35002501

Posada, D. (n.d.). jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution2*, *25*(7), 1253–1256.

Ronquist, F., & Huelsenbeck, J. P. (2003). MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, *19*, 1572–1574.

Rosado, D., & Al, E. (n.d.). Moroccan Herpetofauna: Distribution updates including a DNA barcoding approach. *Herpetozoa*.

Rosenblum, E. (2006). Convergent evolution and divergent selection: lizards at the White Sands ecotone. *The American Naturalist*, *167*(1), 1–15. doi:10.1086/498397

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. *Spring Harbour Press, New York.*

Sandoval, C. P., & Crespi, B. J. (2008). Adaptive evolution of cryptic coloration : the shape of host plants and dorsal stripes in *Timema* walking-sticks. *Biological Journal of the Linnean Society*, *94*, 1–5.

Schleich, H. H., Kästle, W., & Kabisch, K. (1996). *Amphibians and Reptiles of North Africa.* (p. 630 pp.). Biology, Systematics, Field Guide. Koeltz Scientific Books.

Schmitz, A., Brandley, M. C., Mausfeld, P., Vences, M., Glaw, F., Nussbaum, R. a, & Reeder, T. W. (2005). Opening the black box: phylogenetics and morphological evolution of the Malagasy fossorial lizards of the subfamily “Scincinae”. *Molecular Phylogenetics and Evolution*, *34*(1), 118–33. doi:10.1016/j.ympev.2004.08.016

Sinervo, B., Bleay, C., & Adamopoulou, C. (2001). Social causes of correlational selection and the resolution of a heritable throat color polymorphism in a lizard. *Evolution; International Journal of Organic Evolution*, *55*(10), 2040–52. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11761064

Stillwell, C. R., & Fox, C. W. (2009). Geographic variation in body size, sexual size dimorphism and fitness components of a seed beetle: local adaptation versus phenotypic plasticity. *Oikos*, *118*(5), 703–712. doi:10.1111/j.16000706.2008.17327.x

Suárez-Díaz, E., & Anaya-Muñoz, V. H. (2008). History, objectivity, and the construction of molecular phylogenies. *Studies in History and Philosophy of Biological and Biomedical Sciences*, *39*(4), 451–68. doi:10.1016/j.shpsc.2008.09.002

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution*, *30*, 2725–2729.

Uetz, P. (n.d.). *The Reptile Database*. Retrieved from http://www.reptile-database.org/

Valen, L. V. (1965). Morphological variation and width of ecological niche. *The American Naturalist*, *XCIX*(908), 377–390.

Vignieri, S. N., Larson, J. G., & Hoekstra, H. E. (2010). The selective advantage of crypsis in mice. *Evolution; International Journal of Organic Evolution*, *64*(7), 2153– 8. doi:10.1111/j.1558-5646.2010.00976.x

Villesen, P. (2007). FaBox: an online toolbox for fasta sequences. *Molecular Ecology Notes*, *7*(6), 965–968.

Whiting, a. (2003). Phylogenetic relationships and limb loss in sub-Saharan African scincine lizards (Squamata: Scincidae). *Molecular Phylogenetics and Evolution*, *29*(3), 582–598. doi:10.1016/S1055-7903(03)00142-8

APPENDIX 1

HIGH-SALT METHOD OF SALINE EXTRACTION

Needed reagents:

- Lysis buffer (0,5M tris; 0,1M EDTA; 2% SDS; pH 8,0; autoclavated)
- Ammonium acetate (7M; pH 8,0; autoclavated)
- Proteinase K (25 mg/ml)
- Ice-cold isopropanol
- Ice-cold ethanol (70%)
- Ultra-pure water (or other hydration solution)

1. Clean the working bench with bleach and ethanol. In a glass plate, also cleaned with bleach and ethanol, draw separate divisions to avoid sample mixture.
2. Label each eppendorf tube with the corresponding sample codes and pipette 600 µl of lysis buffer to each tube.
3. Using a sterilized scalpel, separate a small amount of tissue sample and cut it in fine pieces. Transfer them into the correspondent eppendorf tube.
4. Add 5-8 µl of proteinase K (depending on the amount of tissue), vortex and incubate at 55ºC overnight.
5. After the tissue is digested, put the tubes in the freezer for 30 minutes.
6. Add 400 µl of ammonium acetate, shake the tubes manually (vortex is not advised as it can damage the DNA), and centrifuge for 20 minutes at 14000 rpm at -4ºC. If precipitated proteins remain in the supernatant, add more 100 µl of ammonium acetate.
7. Label new eppendorf tubes and transfer the supernatant into these, add 600 µl of ice-cold isopropanol and mix, inverting the tubes several times. Put them in the freezer for 3 hours to overnight.
8. Centrifuge for 30 minutes at 14000 at -4ºC and discard the supernatant. It is advised to direct the opening of the tubes to the centre of the centrifuge, since the pellet of DNA will be formed on the opposite side during centrifugation.
9. Add 100 µl of ice-cold 70% ethanol and mix by tapping the bottom of the tube with the finger until the DNA pellet is released. This will wash the DNA from impurities.
10. Centrifuge for 20 minutes at 14000 at -4ºC and discard the supernatant.
11. Let the ethanol evaporate at room temperature or in an incubator for a few hours.
12. When the ethanol has completely evaporated, add 50-200 µl of ultra-pure water (or other DNA hydration solution) and leave to hydrate at room temperature in an agitator for a couple of hours to overnight.